EFFECTS OF SELECTED FOOD COMPONENTS ON INTESTINAL PTEROYLPOLYGLUTAMATE HYDROLASE ACTIVITY: RELATION TO FOLATE BIOAVAILABILITY

BY

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AICAR	aminoimidazole carboxyamide ribonucleotide
BBM	brush border membrane
CM-Sephadex	carboxymethyl-Sephadex
DEAE-Sephadex	diethylaminoethyl-Sephadex
d_2-PteGlu$_6$	[3',5'-$^2H_2$]pteroylhexaglutamic acid
d_4-PteGlu$_1$	[Glu-$^2H_4$]pteroylmonoglutamic acid
EDTA	ethylenediamine tetraacetic acid
FA	folic acid; pteroylmonoglutamic acid
FAICAR	formyl-aminoimidazole carboxyamide ribonucleotide
FBP	folate binding protein
FIGlu	formiminoglutamic acid
5-CH_3-H_4PteGlu$_1$	5-methyltetrahydrofolic acid
5-CHO-H_4PteGlu$_1$	5-formyltetrahydrofolic acid
FDA	Food and Drug Administration
FGAR	formyl-glycinamide ribonucleotide
GAR	glycinamide ribonucleotide
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
H_2PteGlu$_1$	7,8-dihydrofolic acid
H_4PteGlu$_1$ (THF)	5,6,7,8-tetrahydrofolic acid
IU	international unit
LB	lima beans
NMR	nuclear magnetic resonance
OJ	orange juice
*p*ABA	*p*-aminobenzoic acid
*p*ABG	*p*-aminobenzoyl glutamate
*p*ABGlu$_n$	*p*-aminobenzoyl polyglutamate
*p*HMB	*p*-hydroxymercaptobenzoate
PPH	pteroylpolyglutamate hydrolase
PteGlu$_1$	pteroylmonoglutamic acid
PteGlu$_2$	pteroyldiglutamic acid
PteGlu$_3$	pteroyltriglutamic acid
PteGlu$_7$	pteroylheptaglutamic acid
PteGlu$_n$	pteroylpolyglutamic acid
TO	tomato juice

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Major Department: Food Science and Human Nutrition

Dietary folate exists mainly as polyglutamates which require deconjugation by Zn-dependent pteroylpolyglutamate hydrolase (PPH) prior to intestinal absorption. Since deconjugation by PPH is a major factor governing the absorption of dietary folate, factors influencing the deconjugation process may affect folate bioavailability. The present study was conducted to evaluate in vitro inhibition of porcine PPH activity by selected food extracts including orange juice (OJ), tomato juice (TO), and lima beans (LB), and identify the inhibitors from these food extracts. Sixteen percent (v/v) of neutralized orange juice in the reaction mixture caused a complete inhibition, while the same percentage of neutralized tomato juice caused approximately 70% inhibition. Neutralized lima bean homogenates inhibited porcine PPH activity much more weakly. Neutralized filtrates from food extracts by ultrafiltration (MW cutoff 3kD~100kD) had the same inhibitory effect as whole juice. After dialysis of these filtrates, the neutralized retentates (MW >6kD) showed no inhibitory effect. These results indicated that small molecules (MW <6kD) were responsible for this inhibition. Moreover, food extracts were fractionated by ion exchange chromatography and the anionic fraction was found to be inhibitory. By using ion chromatography with UV absorbance and conductivity detectors to identify and quantify the inhibitors, results indicated that citric acid peak was the strong inhibitor in food extracts. Malic acid and phytic acid peaks were weak inhibitors. The inhibition by selected food extracts appeared to be competitive with respect to PteGlu$_3$. The inhibition was not reversed by increasing Zn concentration as high as 700 μM. The results indicated that the inhibition was not mediated by a Zn-chelation process. Folate bioavailability in humans was examined using stable-isotope methods with a single-dose, dual-label protocol. In six trials, adult males (n=7) on a folate saturation regimen (2 mg/d) were given a single 677 nmol oral dose of d$_2$-PteGlu$_6$ and d$_4$-PteGlu$_1$ in foods including OJ, TO, LB, and 52 mM citric acid, using water as the control. Urine was collected for 48 h and the isotopic labeling of urinary folates determined by GC/MS. The d$_2$/d$_4$ isotope ratios of urinary folates were used as criteria for the relative bioavailability of the PteGlu$_1$ and PteGlu$_6$. The d$_2$/d$_4$ ratio in subjects taking orange juice was ~33% less than the control (*P* <0.05). The result suggests that polyglutamyl folates in orange juice exhibit incomplete bioavailability, although by virtue of its high folate concentration, orange juice is an excellent source of available folate.

INTRODUCTION

Folate, one of the water soluble vitamins, is especially concentrated in liver, citrus fruits, dark green leafy vegetables, and yeasts. The biochemical functions of folate are as coenzymes, regulators, and substrate acceptors and donors in one-carbon metabolism. Folate is very important for the rapidly dividing cells, including the epithelium of the intestine, epidermis, and bone marrow (McDowell, 1989). Sauberlich (1990) indicated that there is a relationship between low folate intake and neoplasia in the lung, cervix, and breast. An association between low folate status and arteriosclerosis has been reported by Kang et al. (1987). Folate deficiency is very common in the world (McDowell, 1989; Bailey, 1994) and occurs in approximately 30% of the pregnant women worldwide (Brody, 1991). Factors that increase the need for this vitamin include infancy, adolescence, pregnancy, lactation, and aging. Chronic alcoholism, drug use, and cigarette smoking are often associated with folate deficiency (Bailey, 1990). The deficiency of folate is described by a sequence of events starting with nuclear hypersegmentation of circulating polymorphonuclear leukocytes within two months of deprivation of the vitamin. This is followed by macrocytic anemia, general weakness, depression, and polyneuropathy (Margo et al., 1975; Combs, 1992). Inadequate folate status can cause megaloblastic anemia, low birth weight, and is associated with increased incidence of neural tube defects in newborns (Bailey, 1990).

Dietary folate exists mainly as pteroylpolyglutamates ($PteGlu_n$) which require deconjugation to pteroylmonoglutamates ($PteGlu_1$) by a Zn-dependent pteroylpolyglutamate hydrolase (PPH), associated with the jejunal brush border membrane (BBM), prior to intestinal absorption. This step dictates that folate bioavailability (Gregory et al., 1991 and 1992) varies depending upon PPH level, intestinal pH, cooking procedures, and inhibitors in the food (Retief, 1969). Since deconjugation of pteroylpolyglutamyl folates to the pteroylmonoglutamyl state is a requisite and possibly rate limiting process during absorption, it is important to understand how these forms are digested and absorbed, and what factors affect this process.

Studies with human subjects have indicated that foods differ widely with respect to folate bioavailability (Tamura and Stokstad, 1973; Babu and Srikantia, 1976). Limitations of these studies include low precision and the need for administration of unphysiological large quantities of the test materials. However, a general conclusion of these studies is that the bioavailability of endogenous folates in organ meats including liver and kidney is generally high, while folate bioavailability is variable in other foods (Retief, 1969; Tamura and Stokstad, 1973; Stokstad et al., 1977; Ristow et al., 1982a and 1982b; Abad and Gregory, 1987; Babu and Lakshmaiah, 1987).

The bioavailability of folate from orange juice was previously examined in humans using a variety of experimental designs. Tamura et al. (1976) administrated a single dose of orange juice at several different pHs to folate-saturated human subjects. The bioavailability of the added $PteGlu_7$ was lower than that of the added $PteGlu_1$, which was attributed to an effect of the large volume of orange juice on intestinal pH, thus inhibiting

PPH. In contrast, Rhode et al. (1983) administrated a single daily dose of either orange juice or folic acid (100 μg/d) to folate-restricted women for a 9-wk period. There was no significant difference in serum folate values between the orange juice group and the folic acid group. This finding indicated similar bioavailability of endogenous orange juice folate and supplemental folic acid.

In general, the incomplete availability of folate from a wide variety of legumes has been reported by Babu and Srikantia (1976), Tamura and Stokstad (1973), and Devadas et al. (1979). However, Keagy et al. (1988) reported that California white beans had no effect on the bioavailability of exogenous polyglutamyl folate in human subjects.

These divergent results and other evidence of incomplete bioavailability of dietary folates indicate the need for better understanding of factors affecting the digestion and absorption of folate. To better understand the bioavailability of dietary folate, more complete knowledge is needed with respect to 1) what kinds of food influence PPH activity; 2) what components contained in food extracts influence deconjugation; 3) the mechanism of inhibition; and 4) whether or not in vitro inhibition predicts in vivo effects in human subjects.

Previous studies in this laboratory have indicated that a wide variety of foods including orange juice, tomato juice, and lima beans inhibited human and porcine jejunal brush border PPH activity in vitro (Bhandari and Gregory, 1990). Orange juice and tomato juice, rich in citric acid, had a marked inhibitory effect on porcine PPH activity. Although PPH activity was significantly inhibited by citric acid, knowledge of whether the inhibition was by chelation of zinc ions or other mechanisms is still unknown. Citrus fruit, tomato, and legumes are rich in folates and widely consumed by people. Therefore, it is important to understand how components of these foods affect folate deconjugation during digestion and absorption. Thus, orange juice, tomato juice, and lima beans were chosen as the selected foods used in this study.

Because the PPH from porcine jejunal brush border membrane has similar pH optimum, zinc ion requirements, $PteGlu_n$ affinities, and physiological function to human PPH (Gregory et al., 1987; Bhandari and Gregory, 1990), porcine PPH was used in this study.

The present study was conducted to determine the in vitro effects of various food extracts on porcine PPH activity, and also evaluated the in vivo significance of these food extracts for human subjects. The objectives of this study were as follows:

(1) To assess the relative effect of selected food extracts on porcine PPH activity.
(2) To separate and identify the inhibitors of porcine PPH activity from food extracts.
(3) To evaluate the mode of inhibition of porcine PPH activity by selected food extracts.
(4) To assess the effect of zinc ion on porcine PPH activity.
(5) To evaluate whether the inhibition is mediated by a zinc-chelation process.
(6) To assess the influence of selected foods including orange juice, tomato juice, lima beans, and 52 mM citric acid on the bioavailability of deuterium labeled pteroylmonoglutamyl folate and pteroylpolyglutamyl folate in human subjects.

REVIEW OF THE LITERATURE

History of Folate

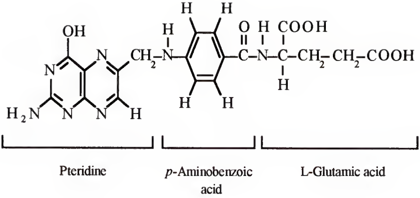
The first clinical description of folic acid deficiency was made by Wills (1931), who described a "tropical macrocytic anemia" that did not have the characteristics of pernicious anemia. This anemia was treated with yeast extract but not with the injectable liver extracts used to treat pernicious anemia (Wills et al., 1937). At the same time, Day et al. (1935) also identified a deficiency syndrome characterized by anemia, leukopenia, ulceration of the gums, and diarrhea. The unknown factors used to treat these symptoms were named "vitamin M" in monkey (Stokstad and Manning, 1938), and "factor U" (Bauernfeind et al., 1938) and "vitamin Bc" in chicks (Hogan and Parrott, 1939). All these conditions in animal species were later found to be responsive to folic acid.

Moreover, it was found that the bacterial "growth factor" present in spinach leaves (Mitchell et al., 1941) and liver (Stokstad, 1943) had many properties in common with the factor in yeast that was necessary to prevent anemia in monkeys and chickens. The active principle was finally isolated and crystallized from liver by Stokstad (1943). The structure and synthesis of pteroylmonoglutamic acid were reported by Angier et al. (1945 and 1946). It was named "folic acid" by Mitchell et al. (1941) who found green vegetables to be excellent sources of this factor. A water-soluble factor necessary for the growth of *Lactobacillus casei* (*L. casei*) was reported by Snell and Peterson in 1940.

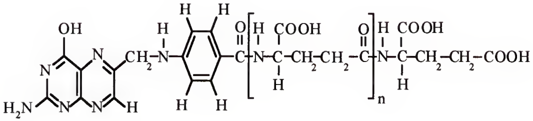
Chemistry of Folate

Folic acid, the common name for pteroyl-L-glutamic acid, is an orange-yellow crystalline substance with a molecular weight (MW) of 441.4. The compound is soluble in water but insoluble in ethanol (Pfiffner et al., 1947; Stokstad and Thenen, 1972). It is unstable to light, acidic or alkaline conditions, and to heat (Stokstad et al., 1948; Stokstad and Thenen, 1972). Folic acid can be reduced Chemically or enzymatically first to 7,8-dihydrofolate ($H_2PteGlu_1$) and then to 5,6,7,8-tetrahydrofolate ($H_4PteGlu_1$), both of which are unstable in an aerobic environment and must be protected by antioxidants including ascorbic acid and 2-mercaptoethanol. Folic acid consists of a pteridine ring joined with *p*-aminobenzoic acid and a single L-glutamic acid molecule (Figure 1). The majority of naturally occurring folate compounds consists of conjugates containing additional L-glutamic acids in γ-peptide linkage (Pfiffner et al., 1946; Scott and Weir, 1976). Natural folates occur in the reduced 7,8-dihydro- and 5,6,7,8-tetrahydro-forms. Folates bearing one-carbon units are 5-methyl-, 10-formyl-, 5-formyl-, 5,10-methenyl, 5,10-methylene-, and 5-formiminotetrahydrofolate.

The UV absorption spectra of the folates are pH-dependent and are characterized by the independent contributions of the pteridine and 4-aminobenzoyl moieties; most have absorption maxima in the region of 280~300 nm (Stokstad and Koch, 1967; Temple and Montgomery, 1984).



(A) pteroylmonoglutamic acid



(B) pteroylpolyglutamic acid

Figure 1. Chemical structures of folates: (A) pteroylmonoglutamic acid and (B) pteroylpolyglutamic acid.

Folate Requirements and Food Sources

The Recommended Dietary Allowance (RDA) for total folate, is 200 μg/d for men and boys over 15 years of age and 180 μg/d for women and girls over 15 years of age (Table 1; Food and Nutrition Board, 1989).

The allowance for pregnant women is increased to 400 μg/d and that for lactating women to 280 μg/d. The data of Sauberlich et al. (1987) were cited as supporting evidence that adult women depleted of folate require 200~250 μg/d of dietary folate to maintain stable plasma levels or to restore them to normal under experimental conditions. This study was one of the most highly controlled and analytically precise experiments of nonpregnant women. The researchers suggested a minimum requirement of 200~250 μg/d of folate and an allowance of 300 μg/d from dietary sources. Therefore, the 200~250 μg would not provide the margin of safety assumed to be provided by an RDA (Bailey, 1992 and 1994).

Folate occurs in a wide variety of foods of both plant and animal origin. Liver, citrus fruits, mushrooms, and dark green leafy vegetables are rich sources of folate in human diets (Perloff and Butrum, 1977). Cooked greens are good sources of folate. However, the folate content may decrease during cooking primarily due to leaching, although thermal and oxidative destruction may also occur. Legumes are another highly rich source of folate. However, legumes contain heat-activated conjugase inhibitors which may decrease the availability of polyglutamyl folates (Krumdieck et al., 1973; Butterworth et al., 1974). Whether these also inhibit jejunal brush border membrane PPH has not been determined.

Table 1. Recommended Dietary Allowances (RDAs) for folate

Category (years)	µg/day	nmol/day
Infant		
0.0~0.5	25	56
0.5~1.0	35	79
Children		
1~3	50	113
4~6	75	170
7~10	100	226
Men		
11~14	150	339
15~18	200	452
19~24	200	452
25~50	200	452
15~18	200	452
over 51	200	452
Women		
11~14	150	339
15~18	180	407
19~24	180	407
25~50	180	407
15~18	180	407
over 51	180	407
Pregnant women	400	904
Lactating women		
First 6 months	280	633
Second 6 months	260	588

Source: Food and Nutrition Board (1989).

The folates in foods are almost exclusively in reduced form, as polyglutamyl derivatives of tetrahydrofolic acid (Table 2). Analyses of foods have revealed a wide distribution of general types of folate derivatives, the predominant forms being 5-methyl-$H_4PteGlu_n$ and 10-formyl-$H_4PteGlu_n$. Folates found in rat liver and kidney are approximately 40% methyl derivatives (Shin et al., 1972; 1974), while those in orange juice and rat red blood cells are exclusively the methyl form (Tamura et al., 1976; Shin et al., 1975). Some plant materials including cabbage and lettuce also contain mainly 5-methyl-$H_4PteGlu_n$ (Chan et al., 1973; Batra et al., 1973).

The analyses also revealed a wide distribution of polyglutamyl folate in foods. Cabbage contains mainly hexa- and heptaglutamates (Chan et al., 1973), while orange juice is 30~40% monoglutamate and 40~50% pentaglutamate (Tamura et al., 1976) and lima beans are approximately 60% pentaglutamate (Seyoum and Selhub, 1993). Organ meats including rat liver and kidney contain mainly pentaglutamates, whereas rat red blood cells contain both penta- and hexaglutamates (Shin et al., 1972 and 1974).

Absorption of Dietary Pteroylpolyglutamates

Dietary folates exist predominantly as polyglutamates (Butterworth et al., 1963) which are hydrolyzed to monoglutamates before their absorption, and entry into the general circulation (Rosenberg, 1981; Halsted, 1979). Therefore, deconjugation is a requisite step in the absorption process (Halsted, 1979). The pteroylpolyglutamate hydrolase (PPH), also termed folate conjugase (EC 3.4.12.10), catalyzes the hydrolysis of poly-γ-glutamyl side chains of $PteGlu_n$. This enzyme has been identified in the intestine

Table 2. Distribution of folate derivatives in various foods and animal tissues

Food	Folate derivatives	Percentage (%)	Reference
Cabbage	5-CH$_3$-H$_4$PteGlu$_7$	51.7	Chan et al. (1973)
	5-CH$_3$-H$_4$PteGlu$_6$	31.7	
	5-CH$_3$-H$_4$PteGlu$_8$	7.4	
	5-CHO-H$_4$PteGlu$_7$	3.0	
Lettuce	5-CH$_3$-H$_4$PteGlu$_5$	34	Batra et al. (1973)
	5-CH$_3$-H$_4$PteGlu$_{4\text{-}2}$	22	
	5-CH$_3$-H$_4$PteGlu$_1$	33	
	5-CHO-H$_4$PteGlu$_1$	11	
Lima beans	5-CH$_3$-H$_4$PteGlu$_5$	22.5	Seyoum and Selhub (1993)
	H$_4$PteGlu$_5$	15.0	
	10-CHO-H$_4$PteGlu$_5$	15.0	
	5-CHO-H$_4$PteGlu$_5$	6.5	
Orange juice	5-CH$_3$-H$_4$PteGlu$_5$	40~50	Tamura et al. (1976)
	5-CH$_3$-H$_4$PteGlu$_1$	30~40	
	5-CH$_3$-H$_4$PteGlu$_{4\text{-}2}$	15~30	
Rat liver	5-CH$_3$-H$_4$PteGlu$_5$	30~40	Shin et al. (1972)
	H$_4$PteGlu$_5$	30~40	
	10-CHO-H$_4$PteGlu$_5$		
	10-CHO-PteGlu$_5$	20~30	
Cow liver	H$_4$PteGlu$_5$	24.2	Seyoum and Selhub (1993)
	5-CH$_3$-H$_4$PteGlu$_1$	17.4	
	H$_2$PteGlu$_1$	17.1	
Rat kidney	5-CH$_3$-H$_4$PteGlu$_5$	36	Shin et al. (1974)
	10-CHO-H$_4$PteGlu$_5$		
	10-CHO-PteGlu$_5$	27	
	H$_4$PteGlu$_1$	25	
	H$_4$PteGlu$_5$	15	
Rat red blood cells	5-CH$_3$-H$_4$PteGlu$_5$	60	Shin et al. (1974)
	5-CH$_3$-H$_4$PteGlu$_6$	35	

of many species, including humans (Hoffbrand and Peters, 1970), chicks (Saini and Rosenberg, 1974), rats (Rosenberg et al., 1969; Elsenhans et al., 1984), guinea pigs (Hoffbrand and Peters, 1969), and pigs (Wang et al., 1985). Hoffbrand and Peters (1970) demonstrated that PPH activity in human intestinal biopsy material is several hundred times greater than in human gastric or pancreatic juice. A cellular fractionation study of guinea pig intestinal mucosa found PPH in the lysosomal fraction with an acid pH optimum (Hoffbrand and Peters, 1969). These studies supported the concept that digestion of dietary folates is an intestinal function and suggested that the hydrolytic process occurs at an intracellular location in the enterocyte.

Two distinct PPH activities have been identified in human jejunal mucosa, a soluble intracellular enzyme and another associated with the brush border membrane (Table 3, Halsted, 1990). Using human jejunal mucosa solubilized in Triton X-100, Reisenauer et al. (1977) separated two PPH activities: one soluble and intracellular, and the other membrane-bound and concentrated in the brush border membrane fraction. Intracellular PPH was optimally active at pH 4.5 and was completely inhibited by the organomercurial *p*-hydroxymercaptobenzoate (*p*HMB), whereas brush border membrane (BBM) PPH was optimally active at neutrality and showed no organomercurial inhibition.

Jejunal brush border membrane PPH was purified about 5,000-fold from human mucosa using sequential Triton X-100 solubilization of the brush border followed by organomercurial affinity chromatography, ion-exchange chromatography, and gel filtration (Chandler et al., 1986). Two polypeptide bands of 115kD and 145kD were observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the purified

Table 3. Comparison of jejunal pteroylpolyglutamate hydrolases in selected mammals

	Intracellular			Brush border membrane			
	Human	Rat	Pig	Human		Pig	
Reference[1]	1, 6	2	3	5	4	3, 5	4
Apparent M_r (kD)	75	80	-[2]	700	-	400	237
pH optimum	4.5	4.5	4.5	6.5	7.0	6.3	7.0
Metal required	None	-	-	Zn^{2+} Co^{2+}	Zn^{2+}[3]	Zn^{2+}	Zn^{2+}[3]
Reducing agent required	Yes	Yes[4]	-	No	-	No	-
*p*HMB[5] inhibition	Yes	Yes	Yes	No	-	No	No
K_m (μM)							
$PteGlu_3$	1.20	0.67	-	0.6	1.9	1.7	1.0
$PteGlu_7$	0.09	0.21	-	0.6	-	-	-
Hydrolysis mechanism	Endo	Endo	-	Exo	Exo	Exo	Exo

Source: Halsted (1990).

[1] Reference : 1. Chandler et al. (1986).
2. Saini and Rosenberg (1974).
3. Gregory et al. (1987).
4. Reisenauer et al. (1977).
5. Wang et al. (1985).
6. Wang et al. (1986).

[2] Dashes represent items not determined.

[3] Activity was measured at pH 7.0 in the presence of 0.1 mM $ZnCl_2$.

[4] The enzyme was purified in the presence of 5 μM mercaptoethanol.

[5] Abbreviations used: *p*HMB: *p*-hydroxymercaptobenzoate; Endo: endopeptidase; Exo: exopeptidase.

enzyme. The purified enzyme had a pH optimum of 6.5. It was activated by Zn^{2+} and Co^{2+}. $PteGlu_2$, $PteGlu_3$, and $PteGlu_7$ were substrates with equal affinity (K_m=0.6 μM) for PPH, whereas $PteGlu_1$ was a weak inhibitor of $PteGlu_3$ hydrolysis (K_i=20 μM). Short-chain Glu_n or components of the pteroyl moiety were non-inhibitory to the enzyme. Human brush border membrane PPH is an exopeptidase. Gregory et al. (1987) verified many of these findings in an analysis of PPH activity in partly purified human jejunal brush border membrane.

Wang et al. (1986) partially purified intracellular PPH from human mucosa. In contrast to human jejunal brush border membrane PPH, this enzyme had an apparent MW of 75kD by gel filtration, a pH optimum of 4.5, and no metal requirement. Intracellular PPH was localized to the intracellular lysosomal fraction. Kinetic studies indicated a K_m of 1.2 μM for $PteGlu_3$. Intracellular PPH exhibited a preference for longer chain $PteGlu_n$; the K_m for $PteGlu_7$ being 0.09 μM. Intracellular PPH, in contrast to jejunal brush border membrane PPH, is an endopeptidase.

The finding of two separate PPH activities in human jejunum contrasted with previous findings of a single intestinal PPH in rat (Elsenhans et al. 1984) and guinea pig (Hoffbrand and Peters, 1969) mucosa; it also raised questions of species differences and the physiological role of each intestinal PPH. Wang et al. (1985) measured intracellular and brush border membrane PPH in rat, monkey, miniature pig, and human mucosa. Brush border membrane PPH was found only in human and pig mucosa; in each species it was stimulated by Zn^{2+} but inhibited insignificantly by *p*HMB. Intracellular PPH was found in all four species; in each instance, it was completely inhibited by *p*HMB but not activated by Zn^{2+}. Similar species differences were observed by Day and Gregory (1984) in a comparison study of the human, pig, and rat mucosal PPH activities. Interestingly, certain characteristics of purified rat jejunal PPH, including its pH optimum and endopeptidase mode of action (Elsenhans et al., 1984), are identical to those described for human jejunal intracellular PPH (Wang et al., 1985).

The respective physiological roles of brush border membrane PPH and intracellular PPH are controversial. The finding of an exopeptidase activity in human brush border membrane PPH by Chandler et al. (1986) and Halsted et al. (1975) constitutes compelling evidence that this enzyme plays the principal role in digesting dietary folates. Intracellular PPH may not be involved in digestion of dietary folates, and instead may serve other functions in enterocyte folate metabolism. However, this hypothesis does not explain the mechanism of folate digestion in species that appear to have no brush border membrane PPH. Alternate absorption processes for species lacking brush border membrane PPH may include pancreatic hydrolysis of $PteGlu_n$ (Arnesjo et al., 1973; Kesavan and Noronha, 1983) or endocytosis of $PteGlu_n$ to an intracellular site of hydrolysis. Bhandari and Gregory (1990) reported that porcine pancreatic PPH can hydrolyze polyglutamyl folates in vitro under mildly acidic conditions similar to those are known to exist in upper small intestine, and it is stable in the presence of other digestive enzymes. However, whether pancreatic PPH acts in vivo in the initial hydrolysis of dietary polyglutamate to compliment the action of intestinal brush border PPH in pigs and human is still unknown.

Transport of Pteroylmonoglutamates

The absorption of folate by the intestine is a multistep process which includes transport through the luminal membrane, a temporary retention and/or metabolism within the enterocyte, and transport through the basolateral membrane. Features of intestinal folate absorption are shown in Table 4 (Mason, 1990).

Since $PteGlu_n$ cannot cross biological membranes, folate is transported to tissues as monoglutamyl folate derivatives in the plasma. The predominant form in portal plasma is the reduced state, tetrahydrofolate. This is taken up by the liver which releases it to the peripheral plasma after metabolic conversion primarily to 5-methyl-$H_4PteGlu_1$ but also to 10-formyl-$H_4PteGlu_1$. The cellular uptake of folate involves a specific, carrier-mediated process that requires energy and Na^+ (Yang et al., 1984; Horne et al., 1978; Selhub and Rosenberg, 1981). Selhub and Rosenberg (1981) indicated that the transport of folates by the intact intestine consists of a saturable component with a luminal pH optimum of 6 and a non-saturable component which predominates at high folate concentrations (>5 μM) and/or high luminal pH.

A folate-binding protein (FBP) is present in the jejunal brush border membrane (Lesile and Rowe, 1972; Selhub et al., 1979; Reisenauer et al., 1986; Shoda, et al., 1990). Shoda et al. (1990) indicated that Scatchard analysis of binding experiments performed at pH 6.0 revealed the existence of two components: one with high affinity (K_d=12~25 nM) and low capacity (V_{max} for acidified BBMs=0.41~0.71 pmol/mg protein) and the other with a low affinity (K_d=1.1~5.1 μM) and high capacity (V_{max} for acidified BBMs=4.05~7.69 pmol/mg protein). Although a role of this protein in folate transport

Table 4. Features of intestinal folate absorption

Feature	Comments
Saturable transport	This implies a carrier-mediated process
Regional specificity	Studies of brush border membrane vesicles suggest that at least some specificity is conferred by the apical membrane rather than by differences in luminal conditions
Acidic pH optimum	Several mechanisms have been proposed
Energy dependence	A direct energy requirement has not yet been conclusively demonstrated
Substrate specificity	Avidity is similar for folic acid, $5\text{-}CH_3\text{-}H_4PteGlu_1$, and methotrexate
Sodium effect	Sodium enhances transport in most preparations, but the mechanism is debated
Intestinal processing of absorbed folate	Folic acid undergoes reduction and one-carbon substitution

Source: Mason (1990).

has been proposed, such a function has not been confirmed. Reisenauer (1988) demonstrated that covalent affinity labeling of the intestinal FBP caused a concomitant loss of folate-binding capacity and folate transport. These results suggest that the FBP is either the transport protein or at least is intimately associated with the transport process (Gregory, 1989).

Transport and accumulation of folate cofactors into the intracellular compartment is followed by their polyglutamation. In this form, one-carbon tetrahydrofolates participate in many biosynthetic processes necessary for normal growth, development, and homeostasis of the organism.

Folate Metabolism and Deficiency

Enzymatic reactions which use folate as coenzymes include 1) certain aspects of purine and pyrimidine synthesis; 2) formation of methionine from homocysteine; 3) metabolism of other amino acids including the interconversion of serine and glycine and the degradation of histidine; 4) oxidation-reduction reactions of in one-carbon metabolism; and 5) formylation reactions (Figure 2. Brody, 1991; Rosenberg and Goodwin, 1971; Pike and Brown, 1975; Kreutler, 1978).

The $PteGlu_1$ that are taken up by cells are trapped as polyglutamate derivatives that cannot cross cell membranes. This is accomplished by the action of the ATP-dependent folylpolyglutamate synthetase, which links glutamyl residues to the vitamin by peptide bonds involving the γ-carboxyl groups. The enzyme requires prior reduction of folate to $H_4PteGlu_1$ or demethylation of 5-methyl-$H_4PteGlu_1$ by vitamin B_{12}-dependent methionine

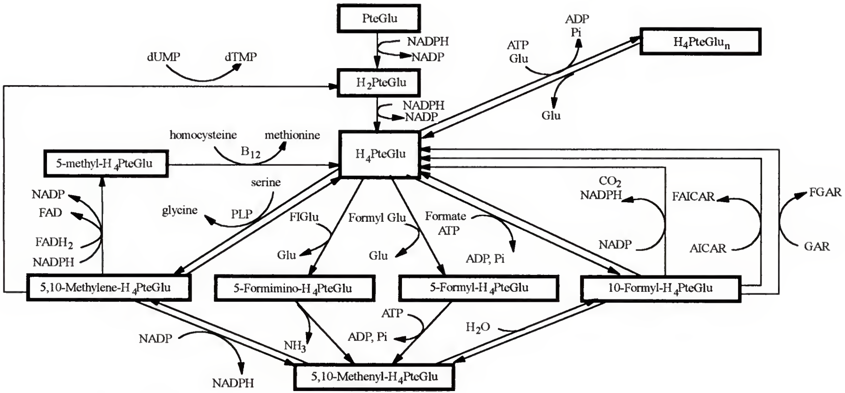


Figure 2. Folate-mediated reactions of one-carbon metabolism (Brody, 1991)

synthetase. $PteGlu_n$ are converted to derivatives of shorter chain-length by cellular conjugases, some of which appear to be Zn-metalloenzymes (Cooper, 1984).

Tissue folates appear to be turned over by the cleavage of the $PteGlu_n$ at the C^9-N^{10} bond to liberate the pteridine and *p*-aminobenzoylpolyglutamate (*p*$ABGlu_n$) moieties. This cleavage may result from chemical oxidation of the cofactor both in intestinal lumen and the tissues. Once formed, *p*$ABGlu_n$ is degraded, presumably by the action of PPH, and *p*ABGlu, *p*-aminobenzoic acid (*p*ABA), and their N-acetyl derivatives are formed (Rodriguez, 1978). Water-soluble side-chain metabolites, *p*$ABGlu_n$ and *p*ABA, are excreted in the urine (Nixon and Bertino, 1972) and bile (Lavoie and Cooper, 1974), which also contains intact folates (Brody, 1991; Combs, 1992).

Folate metabolism consists of reduction of the pteridine ring system, reactions of the polyglutamyl side chain, and acquisition of single-C moieties at certain positions (N^5 or N^{10}) on the pteridine ring. The reduction of the pteridine ring from the two non-reduced states, folic acid ($PteGlu_1$) and 7,8-dihydrofolate ($H_2PteGlu_1$), to the fully reduced form 5,6,7,8-tetrahydrofolate ($H_4PteGlu_1$) that is capable of accepting a single-C unit, is accomplished by the cytosolic enzyme, 7,8-H_2folate reductase. This enzyme is found in high activity in the liver and kidney and in rapidly dividing cells such as tumor cells. The reductase is inhibited by methotrexate. This cancer chemotherapeutic drug exerts its anti-tumor action by inhibiting the reductase activity of tumor cells (Henderson et al., 1986).

Folate deficiency is very common in the world (McDowell, 1989). Deficiencies of folate result in impaired biosynthesis of DNA and RNA and thus, reduced cell division, which is manifest as anemia, dermatologic lesions, and poor growth in most species. The sequence of events in the development of folate deficiency in humans is as follows. Serum folate falls below normal after 3 weeks of folate deprivation. After 7 weeks there is an increase in the average number of lobes of the nuclei of the neutrophils (hypersegmentation). Red blood cell folates gradually fall and reach subnormal levels after 4 months of deprivation. At about 4.5 months the bone marrow becomes megaloblastic and anemia occurs (Herbert, 1962; 1967; and 1968).

Bioavailability of Dietary Folate

The concept of nutrient bioavailability is often poorly defined, conceptually misused, or simply left to the reader to infer a working definition. In the context of folate, bioavailability is most appropriately used to describe the overall efficiency of utilization, including physiological and biochemical processes involved in intestinal absorption, transport, metabolism, and excretion. Thus, it is important to understand the basic chemistry of this vitamin, and the physiology and biochemistry of folate utilization and function, before folate bioavailability can be meaningfully assessed (Gregory, 1994). Reviews concerning folate bioavailability have been published by Rodriguez (1978), Anderson and Talbot (1981), Sauberlich (1985), Bailey (1988), and Gregory (1989). In spite of considerable investigation, the bioavailability of dietary folate still cannot be predicted with accuracy and precision.

Human folate status depends on both the content and bioavailability of the dietary folate. The bioavailability of dietary folate could be affected by dietary factors or physiological conditions influencing human intestinal deconjugation of $PteGlu_n$, intestinal absorption, entry into folate metabolism and enterohepatic circulation, or urinary excretion (Bailey, 1988; Gregory, 1989).

In the folate requirements and bioavailability of naturally occurring dietary folate, Sauberlich et al. (1987) conducted a 92-d controlled metabolic study in women. The subjects received various amounts of folate from dietary sources, added folic acid, or a combination of dietary and added folate. The investigators concluded that a daily intake of 300 µg of total dietary folate was sufficient to meet the folate requirement, which was substantially higher than the intake of folic aicd found to meet the requirement. In comparison to the synthetic folic acid, the response to dietary folate indicated no more than 50% bioavailability. At present this is the most conclusive estimate of the overall bioavailability of folate from food sources.

The bioavailability of folate from foods has been estimated by a number of investigators (Schertel et al., 1965; Retief, 1969; Ghitis and Tripathy, 1970; Butterfield and Calloway, 1972; Tamura and Stokstad, 1973; Grossowitz et al., 1975; Nelson et al., 1975; Babu and Srikantia, 1976; Graham et al., 1980; Ristow et al., 1982a and 1982b; Hoppner and Lampi, 1986; Abad and Gregory, 1987 and 1988; Bhandari and Gregory, 1990). Margo et al. (1975) and Colman et al. (1975) indicated that the bioavailability of folic acid from fortified rice, maize, and bread was significantly lower than that from a solution of folic acid alone, thus suggesting an inhibitory effect of food on folate absorption. Tamura and Stokstad (1973) determined the bioavailability of $H_4PteGlu_1$, 5-formyl-$H_4PteGlu_1$, 5-methyl-$H_4PteGlu_1$, $PteGlu_3$ and $PteGlu_7$, and the bioavailability of folate in 12 test foods fed to folate saturated subjects (Table 5). The results indicated that

Table 5. Apparent bioavailability of endogenous folate in selected foods for human subjects

Food sources	Amount consumed (g)	Apparent bioavailability (%)
Orange juice	600	35[1]
Tomato	1000	37[2]
Banana	533-700	46[2]
Cabbage, cooked	500-700	47[1]
Cabbage, raw	500	47[1]
Liver, cooked, beef	63-94	50[1]
Spinach	200	63[2]
Lima beans, dry, cooked	560	70[1]
Liver, goat	300-500	72[2]
Egg, whole	300-500	72[2]
Banana	890	82[1]
Egg yolk, hard cooked	250	82[1]
Lima beans, frozen, cooked	360	96[1]

[1] Reference: Tamura and Stokstad (1973).
[2] Reference: Babu and Srikantia (1976).

all five $PteGlu_1$ derivatives have bioavailabilities similar to $PteGlu_1$. Folate was highly available in bananas, liver, lima beans, and brewer's yeast. Incomplete folate bioavailability was observed with orange juice, romaine lettuce, egg yolk, and cabbage.

The folate in orange juice exists as 5-methyl-$H_4PteGlu_1$ of varying polyglutamyl chain length. Tamura et al. (1976) administrated a single large dose of orange juice with added $PteGlu_1$ or $PteGlu_7$ to folate saturated subjects. The bioavailability of the added $PteGlu_7$ was lower than that of the added $PteGlu_1$, which was attributed to an effect of the large volume of orange juice on intestinal pH, thus inhibiting PPH. Nelson et al. (1975) used an intraluminal perfusion technique in human subjects to determine the absorption of synthetic folic acid, and folates in orange juice. They reported no significant difference in the amount of folate absorbed from the orange juice or synthetic vitamin solution. Rhode et al. (1983) administered a single daily dose of either orange juice or folic acid (100 μg/d) to women on a folate-restricted diet for a 9-wk period. There was no significant difference in folate status. These findings indicated similar bioavailability of endogenous orange juice folate and supplemental folic acid. Similarly, the apparent bioavailability of orange juice folate has been found to be greater than that of folic acid in rat bioassays (Abad and Gregory, 1987), along with having no inhibitory effect on the utilization of added polyglutamyl folate. However, weak inhibitory effects were observed when [^3H]folic acid or polyglutamyl folates was administered in the presence of orange juice solids in single dose absorption studies in rats (Abad and Gregory, 1988).

The bioavailability of folate in legumes has been reported by Babu and Srikantia (1976), Tamura and Stokstad (1973), and Devadas et al. (1979). A component of legumes which is heat-activated and localized in the skin of the seed has been found to inhibit various PPH (Krumdieck et al., 1973; Butterworth et al., 1974). Tamura and Stokstad (1973), and Babu and Srikantia (1976) reported generally high bioavailability of folate in several legumes, although the precision of the assays was extremely low (Table 5). Similar results were also observed by Devadas et al. (1979). However, Keagy et al. (1988) reported that California white beans had no effect on the bioavailability of exogenous polyglutamyl folate in human subjects. Similar studies were repeatedly performed using [^{3}H]folate in rats (Abad and Gregory, 1988). The presence of cooked red kidney beans caused a small reduction in the bioavailability of both mono- and polyglutamyl folates. These results suggested that beans, like several other dietary components including cabbage or orange juice, exert a weak inhibitory effect on the absorption, rather than deconjugation of folates in this animal model.

Previous studies in this laboratory indicated that a wide variety of foods, including orange juice, tomato juice, and lima bean, inhibited human and porcine PPH in vitro (Bhandari and Gregory, 1990). Orange juice and tomato juice, rich in citric acid, had a marked inhibitory effect on porcine PPH. Enzyme activity was significantly inhibited by citric acid, which may inhibit PPH by chelation of zinc ions. Numerous experimental approaches have been used in the study of folate bioavailability including bioassay with rats, chicks, and human subjects (Babu and Srikantia, 1976; Graham et al., 1980; Keagy and Oace, 1979 and 1984; Tamura and Stokstad, 1973). However, the components of orange juice, tomato juice, and lima beans that inhibit PPH have not been identified.

MATERIALS AND METHODS

Part I: Effects of Selected Food Components on Porcine PPH Activity

Isolation of Porcine Jejunal Brush Border Membrane

Several studies (Gregory et al., 1987; Halsted et al., 1986; Chandler et al., 1986) indicated that PPH in brush border membrane from human and pig jejunal mucosa have similar pH optima, zinc requirement, $PteGlu_n$ affinity, mode of action, and physiological function. As porcine intestine is more easily available than human tissue, we chose to work with porcine PPH. The procurement of pig tissues was approved by the University of Florida Animal Care and Use committee.

Segments of porcine jejunum were obtained at the time of slaughter from healthy, normal adult pigs at the University of Florida Animal Science Department, and stored frozen at -20 °C. The comparison of brush border membrane preparations from frozen and fresh tissues indicated no adverse effects of freezing on PPH activity (Gregory et al., 1987). The porcine jejunal brush border membrane was isolated following the procedures of Selhub et al. (1981). All steps used in the brush border isolation were performed at 4 °C. The lumen of porcine jejunal segments was rinsed with 4 °C 0.9% (w/v) NaCl. A longitudinally opened segment was then placed on a glass plate sitting on ice and the mucosal tissue was scraped with a glass microscope slide. The mucosal tissue was homogenized in 20 volumes of 50 mM D-mannitol for 30 sec at 4 °C with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) setting at high speed. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C. Then, the supernatant was passed through several layers of cheese cloth to remove large particles. Solid $CaCl_2$ was added to the filtrate with 20 min stirring to provide 10 mM $CaCl_2$. The filtrate was centrifuged at 4,000 × *g* for 10 min at 4 °C. The supernatant was removed and recentrifuged at 40,000 × *g* for 15 min. The pellet obtained from this step was resuspended in 5 volumes of isolation buffer (30 mM Tris-HCl, 100 mM NaCl, 0.1 mM zinc acetate, pH 7) and centrifuged at 40,000 × *g* for 15 min. Finally, the pellet was resuspended in isolation buffer. Portions of this preparation were used for protein quantification, alkaline phosphatase activity assay, and PPH activity assays. The remainder portion of this preparation was stored at -20 °C for later analysis.

Protein quantification was performed using the method of Bradford (1976) with bovine serum albumin as standard. Alkaline phosphatase activity was determined using the method of Bessey et al. (1946). The protein quantification and alkaline phosphatase activity were used to estimate the specific activity, yield, and purification fold of PPH.

Enzyme Assay

The standard enzyme assay mixture (pH 7) contained 15 µg porcine jejunal brush border membrane protein, 30 mM Tris-HCl, 100 mM NaCl, 0.1 mM zinc acetate, and 10 µM $PteGlu_3$ which obtained from Dr. B. Schircks in Switzerland with >99% purity as substrate in a total reaction volume of 500 µL. Pteroyltriglutamate has been shown to be as good a substrate for this enzyme as the long-chain polyglutamyl folates (Chandler et al., 1986). The assay system also contained 0.2 mM *p*HMB to inhibit the activity of the intracellular conjugase that may contaminate the brush border fraction (Reisenauer et al., 1977). All assays were performed at 37 °C and then stopped by addition of 50 μL of 50% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 1,000 x *g* for 10 min. In vitro effects of various food extracts were evaluated by including each extract (after neutralization) in a standard enzyme assay mixture.

Initial rates were evaluated for all assays at less than 20% conversion of $PteGlu_3$ to products $PteGlu_2$ and $PteGlu_1$. The rate of reaction was constant up to about 30% hydrolysis of $PteGlu_3$ under these conditions. Rates were linearly related to enzyme concentration. Pteroylpolyglutamate hydrolase (PPH) activity was assayed by an HPLC procedure with postcolumn fluorogenic derivatization as described by Gregory et al. (1984 and 1987). The isocratic mobile phase was 0.05 M potassium acetate buffer (pH 4.9) containing 2% (v/v) acetonitrile, which was pumped at a constant flow rate of 1.5 mL/min through a Perkin Elmer "3×3" C18 column (3 μm octadecylsilyl, 4.6 mm i.d. × 300 mm; Norwalk, CT). A postcolumn solution containing 0.1 M K_2HPO_4, 0.2 M NaCl, and 0.06 mg/mL NaOCl was metered at 0.075 mL/min into the column effluent. The mixture was passed through a 3 m Teflon (0.8 mm i.d.) delay coil at ambient temperature, then into the flow cell of the fluorometer. The FD-300 dual monochromator fluorescence detector (Spectrovision, Inc., Chelmsford, MA) was set with excitation wavelength at 285 nm and emission wavelength 435 nm. Quantification of peaks was performed by electronic integration. Molar fluorescence responses (peak area/μmol) were equivalent for $PteGlu_3$, $PteGlu_2$, and $PteGlu_1$ in this procedure. Pteroyltriglutamate concentrations from 0.2 to 5 μM were added into the enzyme system as substrates to determined the K_m of porcine PPH.

Effect of Zinc Ion on Porcine PPH Activity

Porcine jejunal brush border membrane PPH is a Zn-dependent exopeptidase with neutral pH optimum and apparently equal affinity for $PteGlu_n$ of different chain lengths. The mechanism of inhibition may involve chelation of the zinc ion on Zn-dependent PPH. Therefore, the effect of zinc ion on the porcine PPH was assessed.

The porcine jejunal brush border membrane vesicles were incubated with 1 mM EDTA and 1 mM phenanthroline, followed by dialysis against 30 mM Tris-HCl buffer, pH 7, at 4 °C for 24 h to remove multivalent cations which were bound with EDTA and phenanthroline. Neutralized zinc acetate from 2 to 30 μM was added to ion enzyme assay mixture (30 mM Tris-HCl buffer, pH 7, and 10 μM $PteGlu_3$ as substrate) with 15 μg treated porcine jejunal brush border membrane protein to assess the activation of the apo-enzyme by zinc ions.

Effects of Various Ions on Porcine PPH Activity

Various salts including neutralized NaCl, KCl, $CaCl_2$, and $MgCl_2$ from 20~400 mM were added to the ion enzyme assay mixture (15 μg treated porcine jejunal brush border membrane protein, 30 mM Tris-HCl buffer, pH 7, and 10 μM $PteGlu_3$ as substrate) with and without 0.1 mM zinc acetate to assess the activation of apo-enzyme by other cations.

Preparation of Food Extracts

Extracts of selected foods including orange juice, tomato juice, and lima beans were tested for their effects on porcine PPH activity. Food extracts were diluted in one to one proportion to obtain more concentrated food extracts. The concentration of single strength food extract was considered that the dilution in preparation. Fifty-mL of frozen, concentrated orange juice from Minute Maid® was diluted with 50 mL of the homogenization buffer (30 mM Tris-HCl, 100 mM NaCl, and 0.1 mM zinc acetate, pH 7). Canned diced tomatoes and lima beans from Janet Lee® were drained then 50 g of each were weighed and were finely chopped and diluted with 50 mL of homogenization buffer. The diluted foods were homogenized in a blender setting at high speed for 60 sec then further processed using a Potter-Elvehjem glass-Teflon homogenizer for 60 sec at 4 °C. The food homogenates were then centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was passed through several layers of cheese cloth to remove large particles then adjusted to pH 7 with Tris-base before testing.

Separation of Food Extracts

Food extracts were fractionated through centrifugal ultrafiltration membranes of nominal MW cutoff from 3kD, 10kD, 30kD to 100kD (Centricon microconcentrators, Amicon Corp., Danvers, MA) to collect the various filtrates (Figure 3). A portion of each filtrate was then dialyzed with standard cellulose dialysis tubing (MW cutoff between 6kD~8kD, Spectrum Medical Industries, Inc., Los Angeles, CA) against the homogenization buffer (30 mM Tris-HCl, 100 mM NaCl, and 0.1 mM zinc acetate, pH

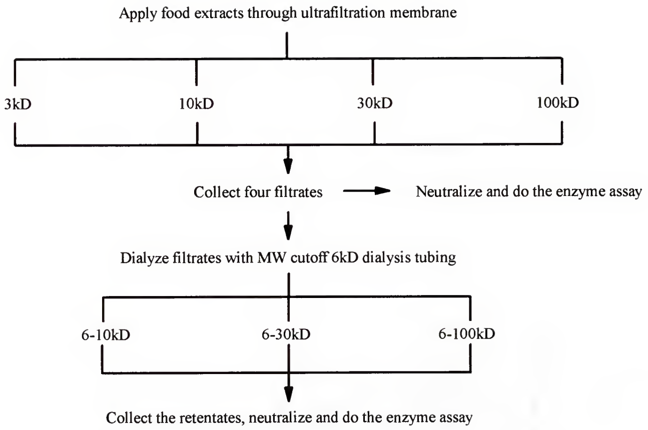

Apply food extracts through ultrafiltration membrane
3kD
10kD
30kD
100kD
Collect four filtrates
Neutralize and do the enzyme assay
Dialyze filtrates with MW cutoff 6kD dialysis tubing
6-10kD
6-30kD
6-100kD
Collect the retentates, neutralize and do the enzyme assay

Figure 3. Experimental design for the separation of porcine PPH inhibitors in food extracts by molecular weight.

7) at 4 °C for 24 h to collect the various retentates.

The neutralized whole juice, various filtrates, and retentates from 0 to 400 μL were added to the standard enzyme assay mixture (15 μg porcine jejunal brush border membrane protein, 30 mM Tris-HCl, 100 mM NaCl, 0.1 mM zinc acetate, and 10 μM $PteGlu_3$ as substrate in a total reaction volume of 500 μL) to evaluate their effects on porcine PPH activity.

The food extracts were also separated into ionic and non-ionic fractions using cation and anion exchange resins (Figure 4). Each food extract was adjusted to pH 4 by 0.1 M HCl and was loaded onto the cation exchanger (Bio Rad AG® 50W-X8 resin or CM-Sephadex). After the non-cationic fraction flowed through and was collected, distilled water was added to remove the remaining non-cationic fraction. NaOH (1 M) was used to elute the cationic fraction from the cationic exchange resin. The cationic fraction was then collected. Both non-cationic and cationic fractions were adjusted to pH 7 with Tris-base and added to the standard enzyme assay mixture to determination their effects on porcine PPH activity. The same procedures were performed with anion exchange resins (Bio Rad AG® 2-X8 resin or DEAE-Sephadex), except that sample pH was adjusted to 7 instead of 4 and 1 M HCl was used to elute the anionic fraction instead of 1 M NaOH.

The ion chromatography method of Bouzas et al. (1991) was used to further separate the inhibitors in food extracts. Analysis was performed using a Bio Rad Aminex® ion exclusion HPX-87H column (9 μm spherical, sulfonated polystyrene-divinylbenzene beads with 8% cross-linking, 7.8 mm i.d. × 300 mm; Bio Rad, Richmond,

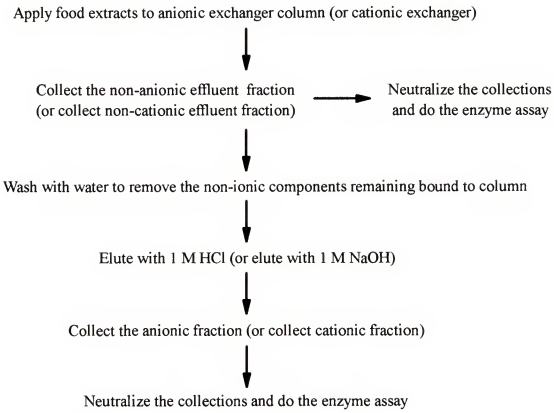

Apply food extracts to anionic exchanger column (or cationic exchanger)
Collect the non-anionic effluent fraction (or collect non-cationic effluent fraction)
Neutralize the collections and do the enzyme assay
Wash with water to remove the non-ionic components remaining bound to column
Elute with 1 M HCl (or elute with 1 M NaOH)
Collect the anionic fraction (or collect cationic fraction)
Neutralize the collections and do the enzyme assay

Figure 4. Experimental design for the fractionation of ionic and non-ionic components of food extracts by ion exchange chromatography.

CA). The isocratic mobile phase was 7 mM H_2SO_4, the flow rate was 0.5 mL/min. The injection volume was 100 µL and detection was by UV absorbance at 210 nm using a Perkin Elmer LC-235 Diode array detector. Each peak was manually collected. The fractions were adjusted to pH 7 with Tris-base and concentrated by using a freeze dryer. After adding distilled water to reconstitute these fractions, the effects of these fractions on porcine PPH activity were evaluated.

Effect of Food Extracts on Porcine PPH Activity

The effects of neutralized food extracts and the fractions separated from food extracts on porcine PPH activity were evaluated. Aliquots (0 to 250 µL) of each extract and fraction were added to the standard enzyme assay mixture (15 µg porcine jejunal brush border membrane protein, 30 mM Tris-HCl, pH 7, 100 mM NaCl, 0.1 mM zinc acetate, and 10 µM $PteGlu_3$ in a total volume of 500 µL). All reactions were conducted at 37 °C and stopped with 50 µL of 50% (w/v) TCA. The effect of the different concentrations of various extracts and separated fractions on porcine PPH activity was assayed by comparing the percent of hydrolysis of $PteGlu_3$ in each test sample to a control without food extracts.

Identification of Inhibitors in Food Extracts

The ion chromatography method of Bouzas et al. (1991) was also used to identify and quantify the inhibitors in food extracts. This separation was performed using a Bio Rad Aminex® ion exclusion HPX-87H column. The isocratic mobile phase was 7 mM

H_2SO_4, which was pumped at 0.5 mL/min. Detection of UV absorbance at 210 nm was accomplished using a Perkin Elmer LC-235 diode array detector. Quantification was relative to organic acid standards including ascorbic acid, citric acid, malic acid, and phytic acid. Tentative identification was performed by comparing the retention time of each peak to the organic acid standards. Then each standard was added to the food extracts to aid in identifying the peaks. Moreover, the ratio of UV absorbance between 210 and 220 nm (A_{210}/A_{220}) of each peak in the food extracts was also determined.

Alkaline phosphatase enzyme assay was used to identify the phytic acid in food extracts. One hundred microliter of food extracts was incubated with 100 µL alkaline phosphatase in buffer solution (100 mM glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$ buffer, pH 10.3, total 500 µL reaction volume) at 37 °C for 30 min. Fifty µL of 0.1 M NaOH was added to stop the reaction. After centrifugation at 1,000 x *g* for 10 min, the supernatant was injected onto a Bio Rad Aminex® ion exclusion HPX-87H column. Comparison of chromatograms before and after alkaline phosphatase treatment showed the disappearance of phytic acid peak with alkaline phosphatase treatment confirmed that phytic acid exists in food extracts.

Ion chromatography with a conductivity detector was also used to identify and quantify the inhibitors in food extracts. Separation was performed using an IonPac anion exchange column (4.0 mm i.d. × 250 mm; AS11 column; 13 µm, ethylvinylbenzene substrate cross-linked with 55% divinylbenzene, and functionalized with alkanol quaternary ammonium groups; Dionex Co., Sunnyvale, CA). The gradient mobile phase was 20% 80 mM NaOH and 80% distilled water pumped at 1 mL/min for 5 min, then linearly changed to 40% 80 mM NaOH and 60% distilled water within 20 min. Conductivity was detected by a Dionex CD20 conductivity detector (Sunnyvale, CA). Quantification was relative to organic acid standards including citric acid, malic acid, and phytic acid. Identification was performed by comparing the retention time of each peak to the organic acid standards.

The GC method of Morrison and Smith (1964) was also used to identify the inhibitors in food extracts. One milliliter of food extracts (or 50 mg standard organic acids including ascorbic acid, citric acid, malic acid, and phytic acid) and 2 mL of 6-7% (v/v) boron trifluoride in methanol were added to a leak-proof Teflon-lined screw cap tube. The tube was tightly capped, well mixed, and then placed in a boiling water bath for 60 min. After cooling the tube with running water, 2 mL of water was added along with 4 mL of ethyl ether. The tube was well mixed and centrifuged at 1,000 x *g* for 5 min to separate the layers. The upper layer was used directly for gas chromatographic analysis.

One microliter of sample was injected into a Perkin Elmer Sigma 3B Gas Chromatograph (Norwalk, CT) equipped with a flame ionization detector and a SE-54 (AT-5) (also called DB-5) column (5% phenyl, 95% methylpolysiloxane; 0.53 mm i.d. x 30 m, and 1.2 µm film thickness; Alltech, Deerfield, IL) attached to a Hewlett-Packard 3393A integrator (Avondale, PA). The carrier gas was helium with a linear velocity of 42 cm/sec. The temperature program was set from 100 °C at 5 °C/min to a final temperature of 200 °C. The temperatures of injector and detector were set at 300 °C.

Mode of Inhibition

The mode of inhibition was evaluated by determining reaction velocity with various concentrations of $PteGlu_3$ as substrate in the presence and absence of neutralized food extract. The Lineweaver-Burk plot, Dixon plot, and Dixon replot were used to identify the mode of inhibition by various food extracts.

Reversibility of Inhibitory Effect on Porcine PPH Activity

The reversibility of inhibition of porcine PPH activity by selected food extracts including orange juice, tomato juice, and lima beans was investigated by increasing zinc acetate from 0.1 mM to 0.7 mM in the reaction.

Part II: Effects of Selected Food Components on Folate Bioavailability in Humans

Dietary folate exists mainly as polyglutamyl folate. Deconjugation of polyglutamyl folates to the monoglutamyl state is a requisite process prior to absorption. Therefore, whatever influences the deconjugation may influence folate absorption. Bhandari and Gregory (1990) indicated that orange juice, tomato juice, lima bean, and citric acid inhibited human and pig PPH activity *in vitro*. The present study was conducted to evaluate the *in vivo* effect in human subjects given orange juice, tomato juice, lima bean, and 52 mM citric acid as the tested foods, and water as control.

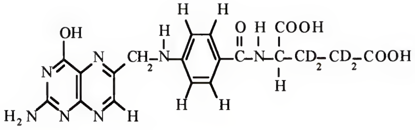
Isotopically Labeled Folate

Gregory and Toth (1988a and 1988b) synthesized and supplied a stable isotopic form of folate that could be accurately analyzed using HPLC and GC/MS methods (Gregory et al., 1990; Gregory, 1990). [3',5'-$^2H_2$]Pteroylhexaglutamate (d_2-PteGlu$_6$) as polyglutamate and [glu-$^2H_4$]pteroylmonoglutamate (d_4-PteGlu$_1$) as monoglutamate (Figure 5) were purified by DEAE-cellulose ion exchange chromatography by Dr. Gregory. The yield from this synthetic procedure was consistently high (98% for d_2 and 90% for d_4). The extent and specificity of labeling of each compound, and the derivatized *p*-aminobenzoylglutamate moieties after cleavage of the C^9-N^{10} bond, was determined by proton nuclear magnetic resonance (NMR) and mass spectrometric analysis (Gregory and Toth, 1988a and 1988b). The d_4-PteGlu$_1$ was labeled with 80% d_4, 15% d_3, and 5% d_0. The d_2-PteGlu$_6$ was labeled with 74.1% d_2, 20.5% d_1, and 5.4% d_0.

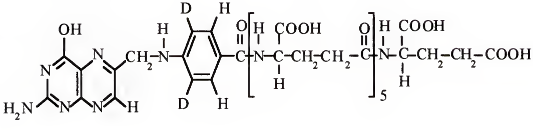
Experimental Protocols with Human Subjects

The subjects (n=7) were adult males aged 20-30 y. All exhibited normal blood chemistry, hematological indices, and serum and erythrocyte folate concentrations. Procedures for selection of subjects and the experimental protocol (Table 6) were approved by the Institutional Review Board of the University of Florida. Informed consent was obtained from each subject.

Because only a small portion of dietary folate is ordinarily excreted into urine, a saturation procedure was used to enhance folate excretion during the isotope studies. During the study period, all subjects consumed their own meals which typically contained



(A) d_4-Pteroylmonoglutamate



(B) d_2-Pteroylhexaglutamate

Figure 5. Deuterium labeled folates: (A) d_4 -pteroylmonoglutamate and (B) d_2-pteroylhexaglutamate.

Table 6. Experimental protocol to determine the effects of selected foods on urinary folate excretion in human subjects.

Day	Duration (d)	Trial	Treatment
1-7	7		10 mg/d d_0-PteGlu$_1$
8-14	7		2 mg/d d_0-PteGlu$_1$[1]
15	1	1	677 nmol d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in 240 mL of water[2]
16-37	21		washout period
38	1	2	677 nmol d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in 240 mL of OJ
39-60	21		washout period
61	1	3	677 nmol d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in 240 mL of TO
62-83	21		washout period
84	1	4	677 nmol d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in 240 mL of LB
85-106	21		washout period
107	1	5	677 nmol d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in 240 mL of 52 mM citric acid
108-129	21		washout period
130	1	6	677 nmol d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in 240 mL of water

[1] From day 8 to day 130, 2 mg/d d_0-PteGlu$_1$ were given to subjects, except on day 15, 38, 61, 84, 107, and 130 of six trials, to have higher recovery of urinary excretion of labeled folates.

[2] During each trial, urine samples were collected for three days (24 h before and 48 h after the labeled dose was given) in three foil-covered 2-L containers containing 3 g solid ascorbic acid.

300~500 μg/d total folate. Each subject was given 10 mg supplemental unlabeled folic acid (d_0-$PteGlu_1$) daily taken in the morning for 7 days and then decreased to 2 mg per day until end of experiment to reach the folate saturated regimen. On each trial day, subjects were given 677 nmol d_2-$PteGlu_6$ and 677 nmol d_4-$PteGlu_1$ instead of d_0-$PteGlu_1$. During each trial, urine samples were collected for three days (24 h before and 48 h after the labeled dose was given) in three foil-covered 2-L containers containing 3 g solid ascorbic acid. Before analysis, samples were flushed with nitrogen gas and stored frozen at -20 °C.

1. Trial 1

After an overnight fast and omission of the morning folic acid tablet, the subjects were given an oral dose of d_2-$PteGlu_6$ (677 nmol) and d_4-$PteGlu_1$ (677 nmol) in 240 mL of water, which was followed by rinsing with 120 mL water.

2. Trial 2

After a 3-wk washout period, the protocol was repeated except for the administration of d_2-$PteGlu_6$ (677 nmol) and d_4-$PteGlu_1$ (677 nmol) in 240 mL of single strength orange juice at its natural pH 4.1.

3. Trial 3

After a 3-wk washout period, the protocol was repeated except for the replacement of water by 240 mL of single strength tomato juice which was homogenized from canned tomatoes at its natural pH 5.0.

4. Trial 4

After a 3-wk washout period, the protocol was repeated except for the replacement of water by 240 mL of single strength lima beans which were homogenized from canned lima beans at its natural pH 6.1.

5. Trial 5

After a 3-wk washout period, the protocol was repeated with d_2-$PteGlu_6$ (677 nmol) and d_4-$PteGlu_1$ (677 nmol) in 240 mL of 52 mM citric acid solution, acidified with HCl to pH 4.1 which was the pH of the orange juice.

6. Trial 6

After a 3-wk washout period, the protocol was repeated with 677 nmol of d_2-$PteGlu_6$ and 677 nmol d_4-$PteGlu_1$ in 240 mL of water instead of citric acid solution. This was a repeat of trial 1 and served as another control.

Blood Collection and Processing Procedure

Fasting morning blood samples were collected in trial 1 (first-term), trial 4 (mid-term), and trial 6 (final-term) to assure that the folate status was normal and consistent during the study. The folate concentrations of serum and whole blood were determined by the microbiological assay procedure of Newman and Tsai (1986). The assay organism for the microbiological assay was *Lactobacillus casei* grown in ATCC 7469 (American Type Culture Collection; Rockville, MD) growth media (Difco Laboratories, Detroit, MI). Precautions were taken to protect samples from light during collection, processing and storage. Blood samples were collected into two 13 mL Vacutainer® tubes (Vacutainer® and Dickinson, Rutherford, NJ). One tube contained EDTA as an anticoagulant and was analyzed for the whole blood folate concentration. Duplicate samples of whole blood were drawn into capillary tubes and centrifuged at 1,000 x *g* for 7 min (IEC MB Centrifuge Micro Hematocrit, International Equipment Co., Needham Heights, MA). Hematocrit values were determined by using a microhematocrit tube reading device and recorded. From the same sample, 0.5 mL portion of whole blood was diluted with distilled water to 5 mL and 1% (w/v) sodium ascorbate was added as a preservative. This whole blood solution was allowed to incubate at room temperature for 30 min. This whole blood preparation was then centrifuged at 1,000 x *g* for 20 min (IEC model HN-SII Centrifuge, International Equipment Co., Needham Heights, MA) to separate blood cells from the plasma, then it was frozen at -20 °C until analyzed. The other silica gel coated tubes (Vacutainer®, and Dickinson, Rutherford, NJ) were held at room temperature for 30 to 60 min to allow time for clotting. After centrifugation at 1,000 x *g* for 20 min, the serum samples were removed and aliquots taken to be analyzed for serum folate concentration. Ascorbic acid (1 mg/mL) was added to aliquots to stabilize serum folate during storage, then the samples were stored at -20 °C.

Falcon® (Becton Dickinson, Rutherford, NJ), 96-well, sterile, flat bottomed, low evaporation, tissue culture plates with lids were used for this microbiological assay. The assay was performed under a laminar flow hood using sterile equipment, supplies and solutions. Three hundred microliters of water was added to the wells that were designated as blanks. One hundred and thirty microliters of ascorbate-phosphate buffer (1 mg L-ascorbic acid in 1 mL of 0.1 M potassium phosphate buffer, pH 6.3) and 20 μL of appropriately diluted standard (1 ng/mL), pooled controls, or samples (1:4 dilution of the serum and 1:40 dilution of whole blood samples) were added in duplicate to selected wells. One hundred and fifty microliters of buffer was dispensed into all other wells of the plate. A total of five serial dilutions of the samples, standard and pooled control were made by using a multi-channel pipette (Costar 8-Pette #04670, Costar Corp., Cambridge, MA). All wells were inoculated with 150 µL of the medium containing the assay organism, and the plates were covered and placed in an incubator at 37 °C for 16 h.

Growth of the microorganism was measured by reading the turbidity of each well at 650 nm using a microtiter plate reader (Molecular Devices UV Max, Menlo Park, CA). Once serum and whole blood folate concentrations were determined, erythrocyte folate concentrations were calculated using the following formula:

$$\text{Red Blood Cell Folate} = \frac{\text{Whole Blood Folate - [Serum Folate x (1-Hematocrit/100)]}}{\text{Hematocrit/100}}$$

Urine Collection and Processing Procedure

Measurement of the isotope distribution in urinary folates was performed by GC/MS. This procedure is based on isolation of urinary folates by affinity chromatography, chemical cleavage of the C^9-N^{10} bond, isolation of the *p*-aminobenzoylglutamate (*p*ABG) fragment by HPLC, and derivatization with trifluoroacetic anhydride and trifluoroethanol. The excretion of each labeled form of urinary folate was calculated from the total daily urine volume, folate concentration, and the ratio of d_2/d_0 and d_4/d_0 (Gregory and Toth, 1988a).

For urinary extraction of folate a column was set up with folate binding protein (FBP) isolated by affinity chromatography column of bovine milk whey and bound to active ester agarose (Affigel 10; BioRad 153-6099). Each affinity chromatography column contained a total of 2 mL of FBP bound to the agarose gel. The binding capacity of the column was 60 nmol of folate would bind to the column. Urine samples (30 mL) were adjusted to pH 7 with 5 M NaOH and then filtered under vacuum through Whatman No. 1 filter paper. Thirty milliliter urine samples were then applied to a 2 mL folate binding protein Affinity column previously equilibrated with 0.1 M potassium phosphate buffer, pH 7. All sample application was conducted at ambient temperature and eluted at 0.3 mL/min as controlled by a peristaltic pump. The column was washed using five 1-mL portions of 25 mM potassium phosphate, pH 7, containing 1 M NaCl, followed by five 1-mL portions of 25 mM potassium phosphate, pH 7. Elution of folates was accomplished using 5 mL of 0.1 M HCl. The first 1 mL of dead volume contained no folate and was discarded and then the eluate was collected into a 5-mL volumetric flask. Sodium ascorbate (10 mg) was immediately added to part of the eluate (1 mL) followed by nitrogen flushing to stabilize the folates for further HPLC analysis. The remaining 4 mL of eluates were flushed with nitrogen gas, then the urinary folates were cleaved and derivatized, as described later, and assayed for the d_2/d_0, d_4/d_0 ratios by GC/MS.

The urinary folate concentration was determined by a minor modification of the HPLC procedure by Gregory and Toth (1988a). Analysis was performed using an Ultrasphere IP column (4.6 mm i.d. × 250 mm, Beckman Instruments, Inc., San Ramon, CA), a mobile phase of 33 mM phosphoric acid, 8% (v/v) acetonitrile at 1 mL/min flow rate, a 100 µL injection volume, and 280 nm absorbance detection by a Perkin Elmer LC-235 diode array detector. Quantification was relative to standards of $PteGlu_1$ and 5-

methyl-H_4PteGlu$_1$, which are two major folate derivatives excreted in urine. The concentration of each standard was spectrophotometrically determined using their molar absorptivity values (Table 7).

After overnight storage in a refrigerator, 0.15 mL of 2 M potassium acetate and 0.15 mL of 1 M NaOH were added to the 4 mL of eluate remaining to give a pH of 5.7. Oxidative cleavage of the C^9-N^{10} bond of purified urinary folates, d_0, d_2, and d_4-PteGlu$_1$ were accomplished using the method of Gregory and Toth (1988a), which yields complete conversion of H_4PteGlu$_1$, 5-methyl-H_4PteGlu$_1$, and PteGlu$_1$ to *p*ABG and pteridine. To oxidize 5-methyl-5,6,7,8-H_4PteGlu$_1$ to 5-methyl-5,6-H_2PteGlu$_1$, 0.22 mL of 5% (v/v) hydrogen peroxide was added for 30 sec, then 0.45 mL 0.1% (w/v) bovine hepatic catalase (Sigma Chemical Co., #C-40) was added to stop the reaction. Samples were acidified by the addition of 0.5 mL of 5 M HCl, then incubated at ambient temperature for 60 min to induce cleavage of 5-methyl-5,6-H_2PteGlu$_1$. Pteroylmonoglutamyl folate was reductively cleaved by addition of 0.27 mL of zinc dust suspension [1 g zinc in 4 mL of 0.5% (w/v) aqueous gelatin], with periodic agitation for 15 min, followed by centrifugation at 1,000 x *g* for 15 min to sediment the zinc. A portion of the sample was centrifuged at 1,000 x *g* for 15 min through a YMT ultrafiltration membrane (Amicon Corp., Danvers, MA) to remove the gelatin. These procedures yield complete disappearance of the parent compounds and quantitative conversion to *p*ABG, as monitored by the HPLC procedure described below. The *p*ABG from the folate cleavage mixtures was isolated by HPLC and monitored at 280 nm by a Perkin Elmer LC-235 diode array absorbance detector. This separation was performed using a preparative

Table 7. Molar absorptivity coefficients of folates

Compound	pH[1]	λ_{max}, nm	$a_{max}x10^{-3}$	Reference
$PteGlu_1$	1	296	18.7	Poe (1977)
	7	282 350	27.0 7.0	Blair et al. (1970)
	13	254 282 363	25.0 23.8 8.7	Poe (1977)
$5-CH_3-H_4PteGlu_1$	1	270 294	24.0 32.2	Blair et al. (1971)
	7	290	32.0	Blair et al. (1971)
	13	290	31.6	Blair et al. (1971)

[1] For pH 1, 0.1 M HCl, for pH 7, phosphate buffer, and for pH 13, 0.1 M NaOH were used, respectively.

column (Partisil M9 10/25 ODS-3, Whatman Inc., Clifton, NJ), a mobile phase of 0.1 M formic acid with 6% (v/v) acetonitrile at 4 mL/min flow rate, and a 5 mL injection volume. The *p*ABG peak was manually collected, evaporated to dryness under a stream of nitrogen at 50 °C, and stored at -20 °C until derivatization.

Mass Spectral Analysis

Vials containing dried *p*ABG fractions were flushed with nitrogen, followed by the addition of 200 µL trifluoroacetic anhydride and 100 µL trifluoroethanol. The samples were tightly capped and placed directly on a 90 °C Reacti-Therm™ heating module (Pierce, Rockford, IL) for 1 h. This derivatization step yielded a trifluoroacetyl-trifluoroethyl-lactam derivative of *p*ABG which retained all isotopic labeling on the *p*ABG moiety of the folates (Gregory and Toth, 1988a). Following equilibration to ambient temperature, excess reagent was evaporated under a stream of nitrogen and the residue dissolved in 50 µL of dry ethyl acetate. The samples were transferred into Target® vials with polyspring inserts (National Scientific Co., Lawrenceville, GA) and stored at -20 °C before GC/MS analysis. All samples were then do GC/MS analysis in triplicate for each vial by Dr. John P. Toth.

Gas chromatography/mass spectrometry (GC/MS) data were acquired using a Hewlett Packard 5890 gas chromatograph (GC) interfaced to a mass spectral selected ion monitored in electron capture negative-ion chemical ionization mode. The derivatized *p*ABG lactams in ethyl acetate solution were injected into a 30 m x 0.25 mm DB5 column (J&W, Folsom, CA) or an equivalent Hewlett Packard HP5 column. The injection port was operated in the splitless mode at a temperature of 250 °C. Injection volumes were typically 1 µL for standards and 3 µL for samples derived from urine extracts. The GC oven was programmed from 200 °C to 300 °C at 20 °C/min with initial and final hold times of 3 min and 1 min, respectively. Electron capture negative ion (ECNI) mass spectra were acquired using methane as the reagent gas at a source pressure of 2.5×10^{-4} Torr. Data were acquired in the selected ion monitoring (SIM) mode at m/z 426, 427, 428, 429, and 430 with a 100 ms dwell time at each m/z value. A series of simultaneous equations was used to calculate the molar ratios of unlabeled and labeled folates (See appendices A and B). The molar ratios of the unlabeled and labeled folates determined by GC/MS, along with the total folate determined by HPLC, were used to calculate the total urinary excretion of unlabeled and labeled folates.

Calculation and Interpretation

Excretion of labeled folates was calculated and expressed as a percentage of the administered dose. For each subject in an individual trial, urinary excretion ratio of pteroylpolyglutamate vs pteroylmonoglutamate was calculated as shown below:

$$d_2/d_4 \text{ excretion ratio} = \frac{\%\text{ of } d_2\text{-PteGlu}_6 \text{ dose excreted as } d_2\text{-folates}}{\%\text{ of } d_4\text{-PteGlu}_1 \text{ dose excreted as } d_4\text{-folates}}$$

Statistical Analysis

The excretion ratios for each trial were used as the primary indicators of bioavailability. Differences between trials with respect to total folate excretion, excretion of d_2 and d_4-labeled folates, recovery of d_2 and d_4-labeled folates, and urinary excretion ratios were compared by using SigmaStat Version 1.0 software (Jandel Co., San Rafael, CA). Factor included in the analysis was differences among control and tested food trials. Means were compared by the Student-Newman-Keuls procedure for one way repeated measures analysis of variance. Differences were considered to be significant at $P < 0.05$ (Steel et al., 1980).

RESULTS

Part I: Effects of Selected Food Components on Porcine PPH Activity

Isolation of Porcine Jejunal Brush Border Membrane

On the basis of the marker enzyme alkaline phosphatase, the enrichment of porcine jejunal brush border membrane (BBM) vesicles protein following purification was more than seven fold relative to the original homogenate (Table 8). The specific activity of alkaline phosphatase was 2.36 units per mg porcine jejunal brush border membrane vesicles protein (unit=µmol/min) (Table 8). The protein concentration of porcine jejunal brush border membrane vesicles were 5.05 mg/mL and the yield of porcine jejunal brush border membrane vesicles PPH were approximately 43.6% (Table 8). The percentage hydrolysis of PPH of $PteGlu_3$ did not change when assayed with or without 0.2 mM *p*HMB, which indicated no existence of intracellular PPH associated with the porcine brush border membrane vesicles. Intact porcine jejunal brush border membrane vesicles were used as a source of PPH activity in all experiments to mimic the body's intestinal environment.

For all assays of porcine PPH activity the initial rates were evaluated at less than 20% conversion of $PteGlu_3$ to products $PteGlu_2$ and $PteGlu_1$. The rate of reaction was constant up to approximately 30% hydrolysis of $PteGlu_3$ to $PteGlu_2$ and $PteGlu_1$ under these conditions. In the enzyme assay the substrate $PteGlu_3$ and products $PteGlu_2$ and

Table 8. Purification of pteroylpolyglutamate hydrolase from pig jejunum

	Supernatant from first centrifuge	Supernatant after adding $CaCl_2$	Pellet from ultracentrifuge	Final BBM vesicles
Total volume (mL)	150	120	15	10
Protein conc. (mg/mL)	5.73	2.58	4.67	5.05
PPH conc. (units[1]/mL)	1.83	2.10	10.4	11.9
Total units (units)	274	252	155	119
Specific activity (units/mg)	0.319	0.815	2.22	2.36
Yield (%)	100	92.0	56.7	43.6
Purification fold	1.0	2.6	7.0	7.4

[1] Specific activity of marker enzyme alkaline phosphatase is expressed unit/mg (=µmol/min/mg porcine jejunal BBM vesicle protein).

PteGlu_1 were adequately separated by HPLC. A typical HPLC chromatogram of PteGlu_3, PteGlu_2, and PteGlu_1 is shown in Figure 6.

Figure 7 illustrates that porcine jejunal brush border membrane vesicles exhibited high PPH activity toward the PteGlu_3 substrate. Michaelis-Menten and Lineweaver-Burk plots indicated that the K_m=1.02±0.02 μM (Mean±SD) for this enzyme and substrate. In the enzyme assay 10 μM PteGlu_3, ten times the K_m value, was included to the standard assay mixture (15 μg porcine jejunal brush border membrane protein, 30 mM Tris-HCl, 100 mM NaCl, 0.2 mM *p*HMB, pH 7) as substrate.

Effect of Zinc Ion on Porcine PPH Activity

PPH activity was eliminated by treatment with EDTA and phenanthroline but was fully restored by adding zinc acetate (2~30 μM) to the ion enzyme assay mixture (15 μg treated porcine jejunal brush border membrane vesicle protein, 30 mM Tris-HCl buffer, pH 7, and 10 μM PteGlu_3 as substrate) (K_m=11.8±1.4 μM for Zn^{2+}) (Figure 8). In the enzyme assay 100 μM zinc acetate, ten times the K_m value, was added to the standard assay mixture to assure sufficient zinc.

Effect of Various Ions on Porcine PPH Activity

PPH activity was eliminated by treatment with EDTA and phenanthroline and was not restored by adding NaCl (20~400 mM), KCl (20~400 mM), $MgCl_2$ (20~100 mM), or $CaCl_2$ (20~100 mM) to the ion enzyme assay mixture (15 μg treated porcine jejunal brush border membrane vesicle protein, 30 mM Tris-HCl buffer, pH 7, and 10 μM

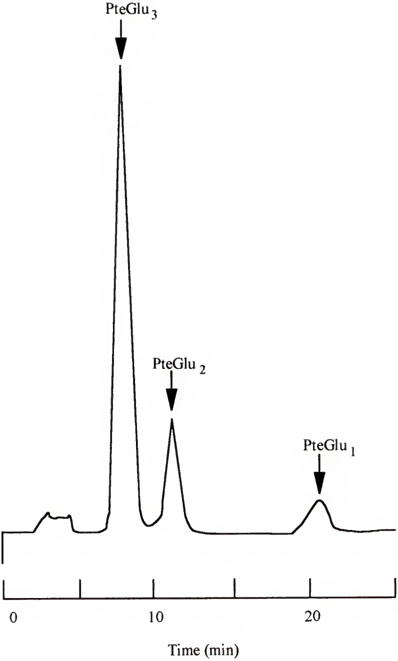
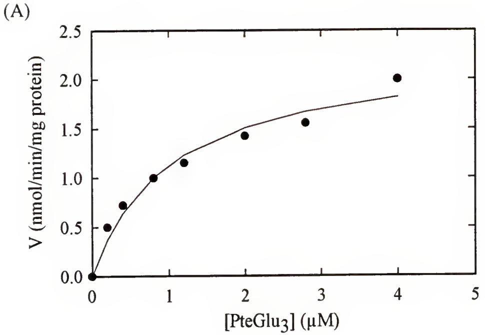


Figure 6. Representative chromatogram of HPLC analysis of typical porcine PPH reaction mixture with post-column fluorogenic derivatization. Initial substrate PteGlu_3 concentration was 10 μM.



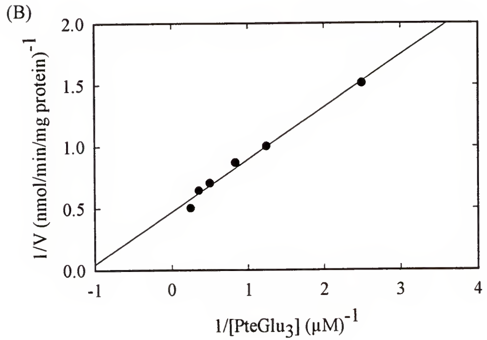


Figure 7. Determination of K_m for $PteGlu_3$ with porcine PPH by (A) Michaelis-Menten plot and (B) Lineweaver-Burk plot.

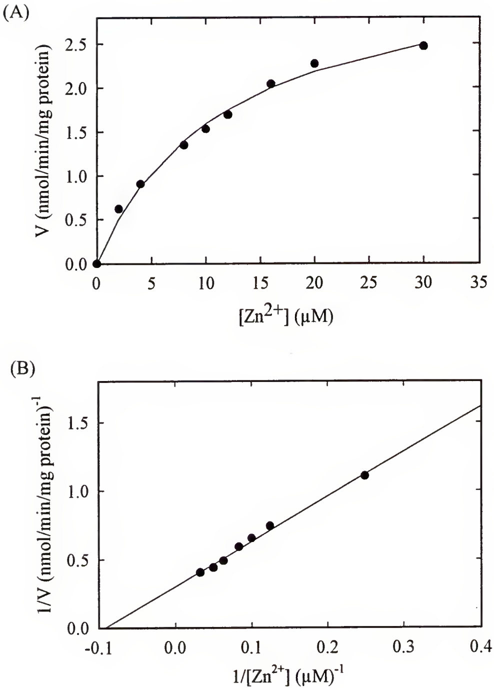


Figure 8. Activation of EDTA-treated porcine PPH by zinc: (A) Michaelis-Menten plot and (B) Lineweaver-Burk plot.

$PteGlu_3$ as substrate) (Data not shown). PPH activity was inhibited by 300~400 mM NaCl when incubated with 100 μM zinc acetate in the ion assay mixture (Data not shown).

Effect of Food Extracts on Porcine PPH Activity

Food extracts, including orange juice, tomato juice, and lima bean homogenate, were separated by molecular weight. The neutralized whole juices, various filtrates (3kD, 10kD, 30kD, and 100kD), and retentates (6~10kD, 6~30kD, and 6~100kD) were evaluated for their effects on porcine PPH activity.

Neutralized whole orange juice (OJ) as well as the 3kD, 10kD, 30kD, and 100kD filtrate fractions exhibited equivalent inhibition of porcine PPH activity. Approximately 16% (v/v) orange juice completely inhibited porcine PPH activity in the standard assay. The retentate fractions (6~10kD, 6~30kD, and 6~100kD) did not significantly inhibit PPH (Figure 9). Figures 10 and 11 illustrate that approximately 48% (w/v) tomato juice (TO) and 78% (w/v) lima bean homogenate (LB) completely inhibited porcine PPH activity. The retentate fractions (6~10kD, 6~30kD, and 6~100kD) in both tomato juice and lima bean homogenate did not significantly inhibit porcine PPH activity.

Thus, the major compounds responsible for in vitro inhibition of porcine PPH were associated with the low MW fraction (MW <6kD). Since the exact inhibitory components are not yet known, % (v/v or w/v) was used instead of molar concentration.

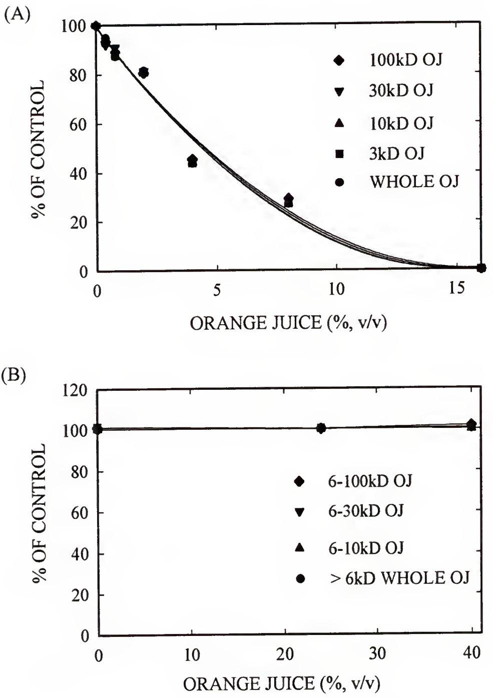


Figure 9. Effects of concentration (%, v/v) of orange juice on porcine PPH activity: (A) various filtrates (3kD, 10kD, 30kD, 100kD) and (B) various retentates (6~10kD, 6~30kD, 6~100kD).

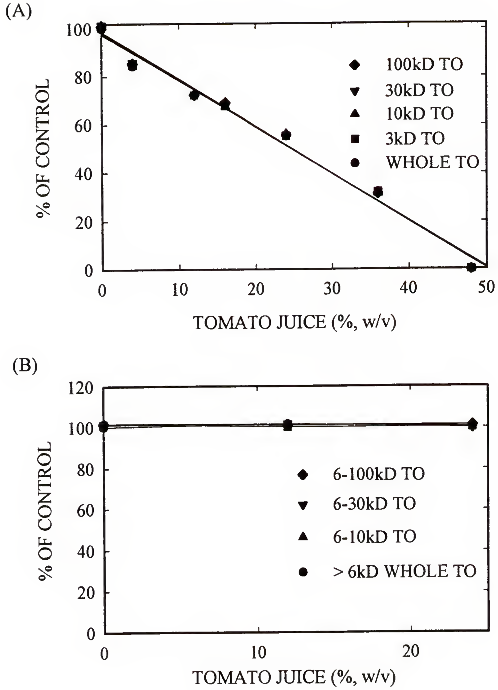


Figure 10. Effects of concentration (%, w/v) of tomato juice on porcine PPH activity: (A) various filtrates (3kD, 10kD, 30kD, 100kD) and (B) various retentates (6~10kD, 6~30kD, 6~100kD).

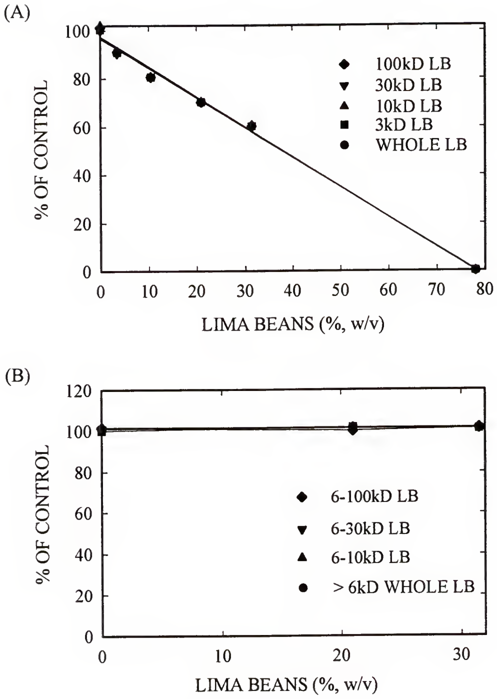


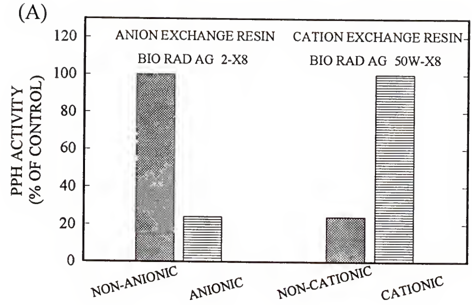
Figure 11. Effects of concentration (%, w/v) of lima beans on porcine PPH activity: (A) various filtrates (3kD, 10kD, 30kD, 100kD) and (B) various retentates (6~10kD, 6~30kD, 6~100kD).

Effects of Ionic Components on Porcine PPH Activity

Anionic fractions of orange juice, tomato juice, and lima bean extracts, separated by ion exchange chromatography, exhibited inhibitory effects on porcine PPH activity (Figures 12, 13, and 14). The same amount (30 µL) of each anionic fraction of these extracts was added to the enzyme system and showed different inhibitory effects on porcine PPH activity. The anionic fraction of 3% (v/v) single strength orange juice exhibited 24% of control PPH activity which indicated that the anionic fraction in orange juice had a strong inhibitory effect on porcine PPH activity. Twelve percentage (w/v) of single strength tomato juice and lima bean homogenate showed 73% and 80% of control PPH activity, which indicated that anionic fractions in tomato juice and lima beans only moderately inhibited porcine PPH activity. The cationic fractions of these food extracts did not significantly inhibit porcine PPH activity.

Effects of Selected Organic Acids on Porcine PPH Activity

The selected organic acids including citric acid, malic acid, and phytic acid were adequately separated by an Aminex® ion exclusion HPX-87H column as monitored with UV detection (Figure 15). Typical HPLC chromatograms of food extracts using this same column are shown in Figure 16. All these peaks were collected, concentrated, and then the same amount (30 µL; same amount of the ionic fraction added in enzyme system as described above) of each food extract was added to the enzyme system. The results of the enzyme assay indicated that the citric acid peak had a strong inhibitory effect on porcine PPH activity. The malic acid peak had a moderate inhibition, while the phytic



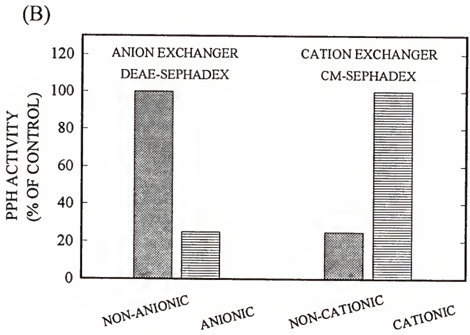
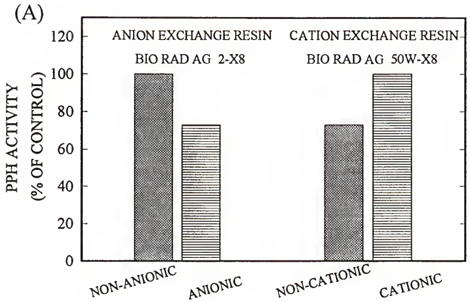


Figure 12. Effects of the fractions of orange juice from ion exchange chromatography on porcine PPH activity: (A) Bio Rad AG® 2-X8 and AG® 50W-X8 and (B) DEAE- and CM-Sephadex.



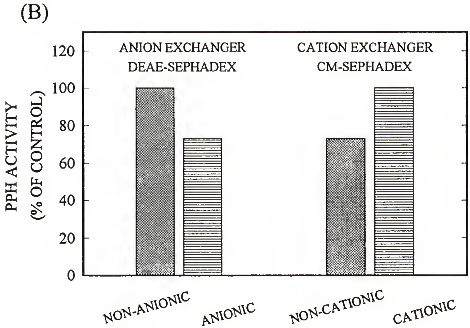


Figure 13. Effects of the fractions of tomato juice from ion exchange chromatography on porcine PPH activity: (A) Bio Rad AG® 2-X8 and AG® 50W-X8 and (B) DEAE- and CM-Sephadex.

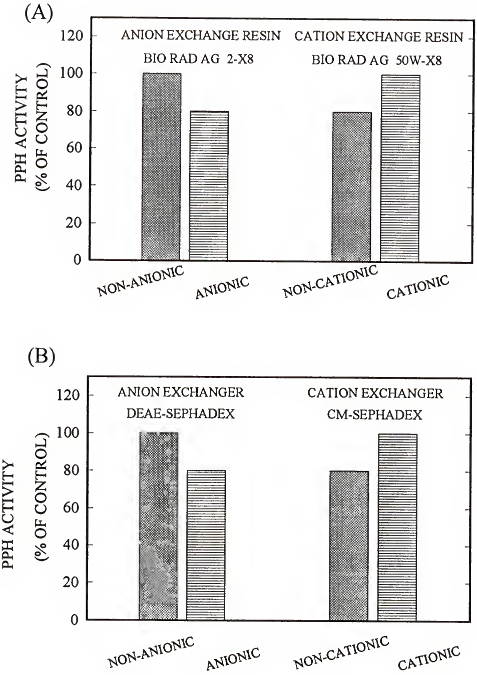


Figure 14. Effects of the fractions of lima beans from ion exchange chromatography on porcine PPH activity: (A) Bio Rad AG® 2-X8 and AG® 50W-X8 and (B) DEAE- and CM-Sephadex.

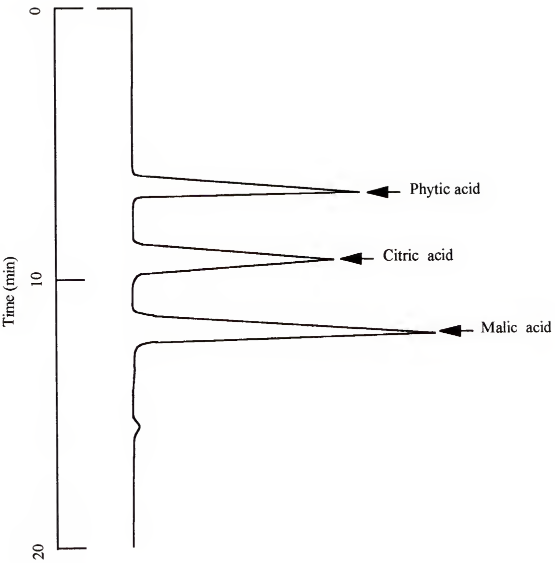


Figure 15. Representative chromatogram of HPLC anaylsis of organic acid with UV 210 nm detection. The concentrations of these organic acids were 0.01% (w/v).

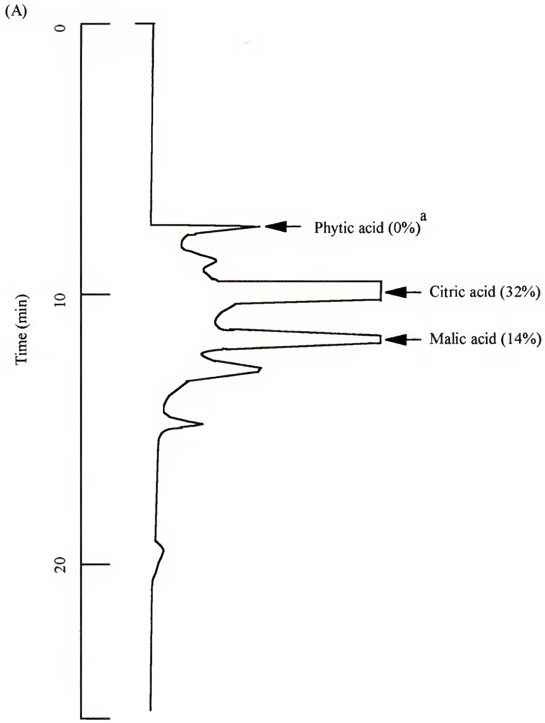


Figure 16. Typical HPLC chromatograms of food extracts using an anion exchange column: (A) orange juice, (B) tomato juice, and (C) lima bean. [a] Number in ( ) expressed the % of inhibition was due to that organic acid peak.

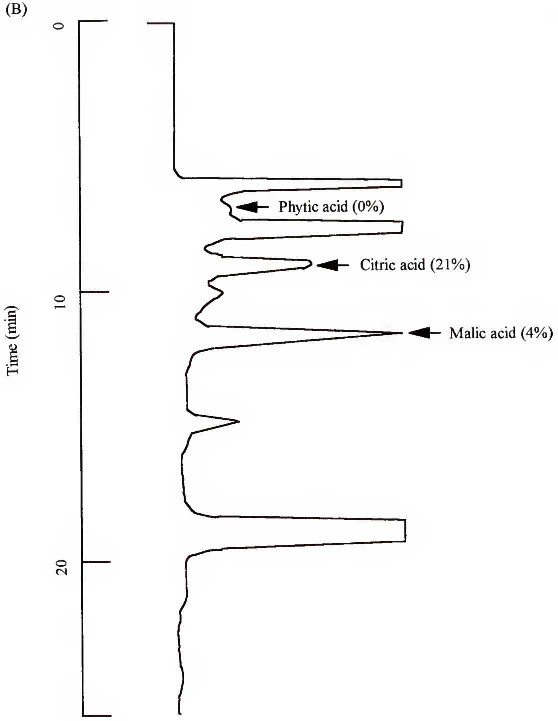


Figure 16. -- Continued.

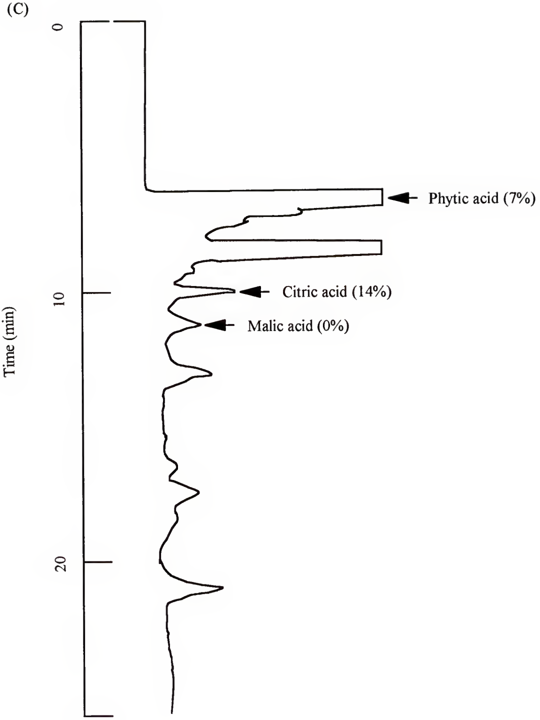


Figure 16. -- Continued.

acid peak had a weak inhibition on porcine PPH activity. When 75% porcine PPH activity was inhibited by 3% (v/v) of single strength orange juice, 32% of the inhibition was due to citric acid peak, 14% of the inhibition was due to malic acid peak, the remaining 22% was due to unknown compounds. None of the inhibition was due to phytic acid peak. When 30% porcine PPH activity was inhibited by 12% (w/v) of single strength tomato juice, 21% of the inhibition was due to citric acid peak, 4% of the inhibition was due to malic acid peak, the remaining 5% was due to unknown compounds, with no inhibition by phytic acid peak. When 24% porcine PPH activity was inhibited by 12% (w/v) of single strength lima beans, 14% of the inhibition was due to citric acid peak, 7% of the inhibition was due to phytic acid peak, the remaining 3% was due to unknown compounds, with no inhibition by malic acid peak.

Identification of Inhibitors in Food Extracts

The ion chromatography method of Bouzas et al. (1991) was used to identify and quantify the inhibitors in food extracts. Identification was assured by comparing the retention time of each peak to the organic acid standards and by spiking each food extract with the standards. Moreover, the ratio of UV absorbance between 210 and 220 nm (A_{210}/A_{220}) of each peak in the food extracts was used to identify it by comparing the ratio to the standards. The comparison of the A_{210}/A_{220} ratio of standard organic acids, including phytic acid, citric acid, and malic acid, to food extracts including orange juice, tomato juice, and lima beans is shown in Table 9. Tomato juice, lima bean homogenate and standard citric acid had similar A_{210}/A_{220} ratios which indicated that citric acid peaks

Table 9. Comparison of A_{210}/A_{220} ratio of organic acids in food extracts

	Phytic acid	Citric acid	Malic acid
Standard	1.14±0.014	1.38±0.011	1.41±0.012
Orange juice	1.60±0.012	1.44±0.012	1.00±0.011
Tomato juice	1.83±0.012	1.37±0.011	1.63±0.013
Lima beans	1.36±0.015	1.39±0.011	1.50±0.012

in tomato juice and lima beans were more pure than the same peak in orange juice. Phytic acid and malic acid peaks were not pure in orange juice, tomato juice, and lima bean extract by comparing the A_{210}/A_{220} ratios between the standard and these food extracts.

The selected organic acids, including citric acid, malic acid, and phytic acid were adequately separated by an IonPac ion chromatography AS-11 column using conductivity detection (Figure 17). The identification and quantification of selected organic acids in food extracts by conductivity are shown in Table 10. Single strength orange juice contained high amounts of citric acid and malic acid (1.18%, v/v = 61.4 mM; 0.64%, v/v = 47.7 mM), while single strength tomato juice contained moderate amounts of citric acid and malic acid (0.69%, w/v = 35.9 mM; 0.22%, w/v = 16.4 mM). Lima bean homogenate contained low of citric acid and malic acid (0.33%, w/v = 17.2 mM; 0%), but a high amount of phytic acid (0.764%, w/v = 11.6 mM). Single strength tomato juice and single strength orange juice contained low amounts of phytic acid (tomato juice 0.245%, w/v = 3.71 mM; orange juice 0.121%, v/v = 1.83 mM).

Previous results indicated that citric acid caused strong inhibition of porcine PPH activity, while malic acid and phytic acid caused only weak inhibitory effects on porcine PPH activity. All these three organic acids were derivatized for detection by gas chromatography; only citric acid could be detected. The GC chromatogram of citric acid is shown in Figure 18.

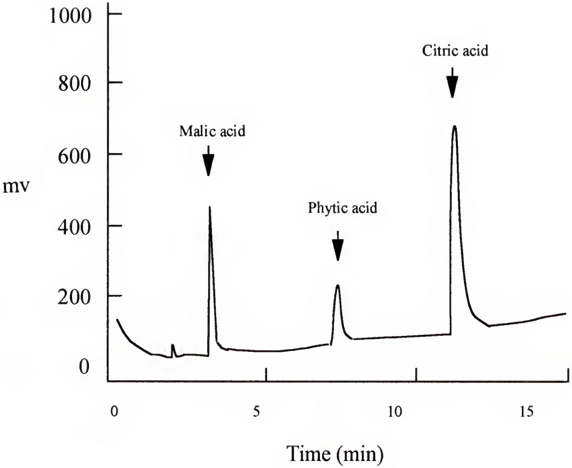


Figure 17. Typical chromatogram of organic acids using an IonPac chromatography AS-11 column as monitored by conductivity.

Table 10. Identification and quantification of organic acids in food extracts by conductivity

	Phytic acid		Citric acid		Malic acid	
	%[1]	mM	%	mM	%	mM
Orange juice	0.121	1.83	1.18	61.4	0.64	47.7
Tomato juice	0.245	3.71	0.69	35.9	0.22	16.4
Lima beans	0.764	116	0.33	17.2	0	0

[1] Percentage is expressed g/100 mL for single strength orange juice; g/100 g for single strength tomato juice and lima beans.

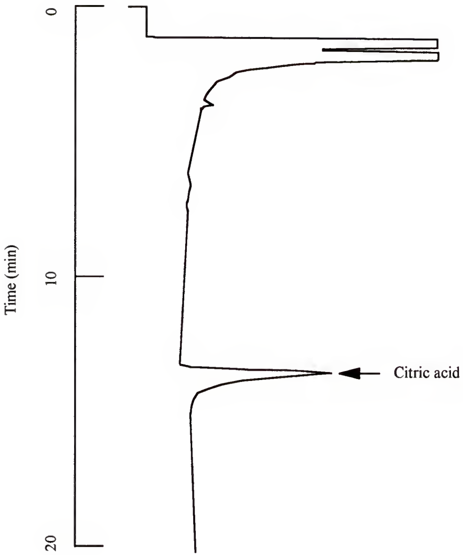


Figure 18. Gas chromatogram of citric acid.

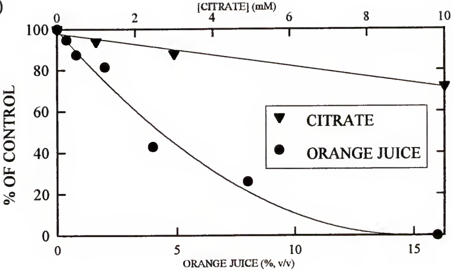
Mode of Inhibition

Effects of increasing the percentage (v/v or w/v) of selected food extracts, orange juice, tomato juice, and lima beans, and their equivalent concentrations of pure organic acids (phytic acid, citric acid, and malic acid) on porcine PPH activity are shown in Figures 19, 20, and 21. Comparison of the curves shown in Figure 19 indicates that for orange juice, only 24% of the inhibition was due to citric acid, 18% was due to malic acid, 5% was due to phytic acid, and the remaining 53% was not determined. Comparison of the curves shown in Figure 20 indicates that for tomato juice, only 40% shown in Figure 20 indicates that for tomato juice, only 40% of the inhibition was due to citric acid, 15% was due to malic acid, 10% was due to phytic acid, and the remaining 35% was due to unknown compounds, assuming no interactive effects. Comparison of curves shown in Figure 21 indicates that for lima beans, only 40% of the inhibition was due to citric acid, 18% was due to phytic acid, and the remaining 45% was due to unknown compounds. Since there is no malic acid exists in lima beans, no inhibition could be attributed to malic acid.

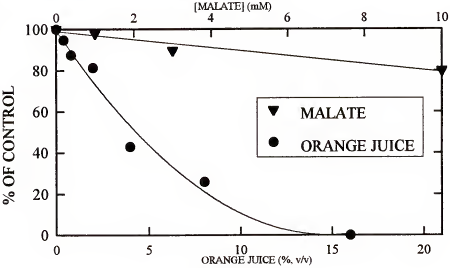
The mode of inhibition was evaluated by determining reaction velocity with various concentrations of substrate PteGlu$_3$ in the presence and absence of neutralized food extracts. Inhibition of porcine PPH activity appeared to be competitive with respect to PteGlu$_3$ not only by selected food extracts including orange juice [K_i=2.14% (v/v) single strength OJ], tomato juice [K_i=5.00% (w/v) single strength TO], and lima beans [K_i=14.7% (w/v) LB] (Figure 22~24), but also by selected organic acids including ascorbic acid (K_i=19.6 mM), citric acid (K_i=6.42 mM), malic acid (K_i=10.1 mM), and phytic acid

Figure 19. Effects of increasing the percentage (v/v) of single strength orange juice and its equivalent concentration of pure organic acids on porcine PPH activity: (A) citric acid, (B) malic acid, and (C) phytic acid.

(A)


[CITRATE] (mM)
0
2
4
6
8
10
100
80
60
40
20
0
% OF CONTROL
CITRATE
ORANGE JUICE
0
5
10
15
ORANGE JUICE (%, v/v)

(B)


[MALATE] (mM)
0
2
4
6
8
10
100
80
60
40
20
0
% OF CONTROL
MALATE
ORANGE JUICE
0
5
10
15
20
ORANGE JUICE (%, v/v)

(C)

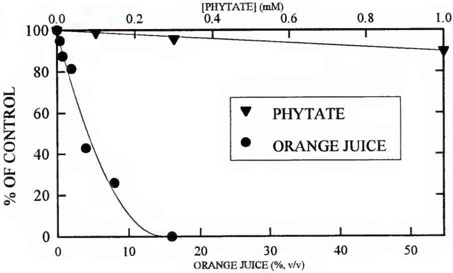

[PHYTATE] (mM)
0.0
0.2
0.4
0.6
0.8
1.0
100
80
60
40
20
0
% OF CONTROL
PHYTATE
ORANGE JUICE
0
10
20
30
40
50
ORANGE JUICE (%, v/v)

Figure 20. Effects of increasing the percentage (w/v) of single strength tomato juice and its equivalent concentration of pure organic acids on porcine PPH activity: (A) citric acid, (B) malic acid, and (C) phytic acid.

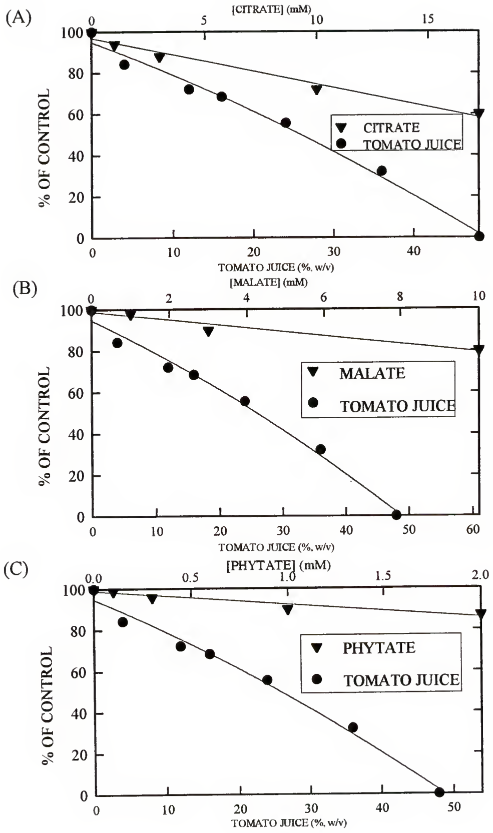
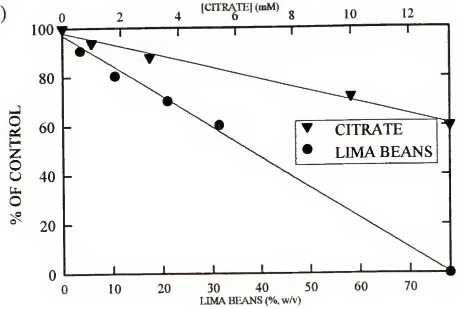

(A)
[CITRATE] (mM)
0
5
10
15
100
80
60
40
20
0
% OF CONTROL
CITRATE
TOMATO JUICE
0
10
20
30
40
TOMATO JUICE (%, w/v)
(B)
[MALATE] (mM)
0
2
4
6
8
10
100
80
60
40
20
0
% OF CONTROL
MALATE
TOMATO JUICE
0
10
20
30
40
50
60
TOMATO JUICE (%, w/v)
(C)
[PHYTATE] (mM)
0.0
0.5
1.0
1.5
2.0
100
80
60
40
20
0
% OF CONTROL
PHYTATE
TOMATO JUICE
0
10
20
30
40
50
TOMATO JUICE (%, w/v)

Figure 21. Effects of increasing the percentage (w/v) of single strength lima beans and its equivalent concentration of pure organic acids on porcine PPH activity: (A) citric acid and (B) phytic acid.

(A)


[CITRATE] (mM)
0
2
4
6
8
10
12
100
80
60
40
20
0
% OF CONTROL
CITRATE
LIMA BEANS
0
10
20
30
40
50
60
70
LIMA BEANS (%, w/v)

(B)

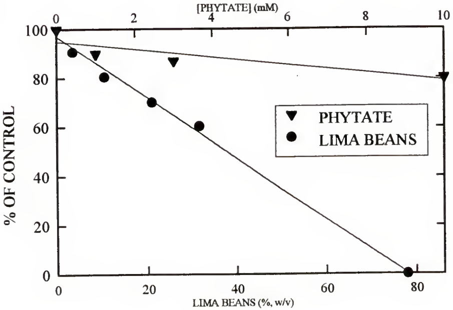

[PHYTATE] (mM)
0
2
4
6
8
10
100
80
60
40
20
0
% OF CONTROL
PHYTATE
LIMA BEANS
0
20
40
60
80
LIMA BEANS (%, w/v)

Figure 22. Inhibitory effect of orange juice on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.

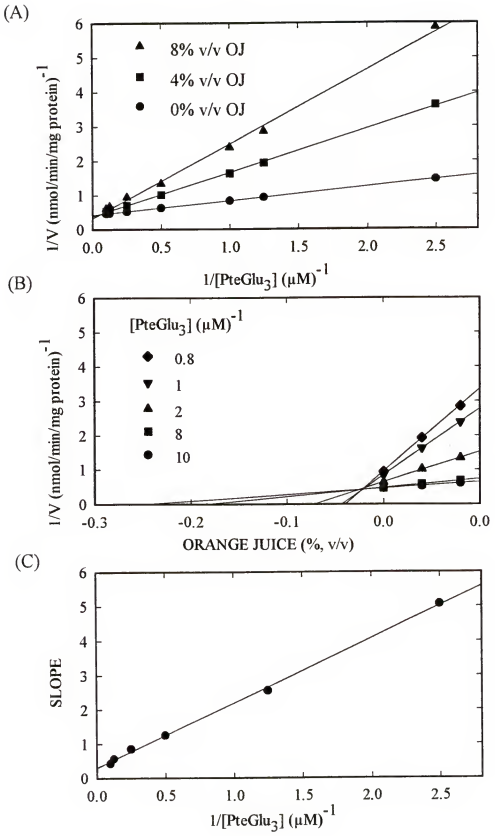
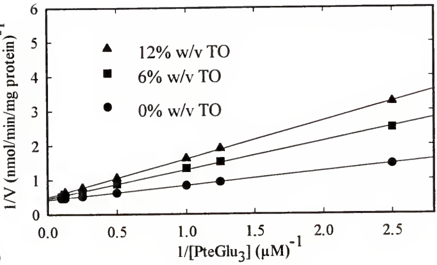
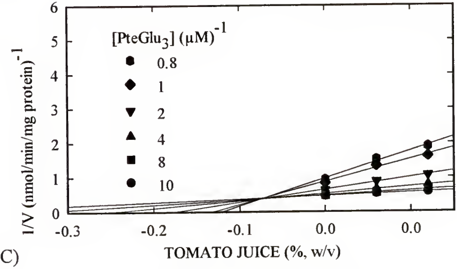

(A)
8% v/v OJ
4% v/v OJ
0% v/v OJ
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$
(B)
[PteGlu$_3$] (μM)$^{-1}$
0.8
1
2
8
10
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
-0.3
-0.2
-0.1
0.0
0.0
ORANGE JUICE (%, v/v)
(C)
SLOPE
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

Figure 23. Inhibitory effect of tomato juice on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.

(A)


12% w/v TO
6% w/v TO
0% w/v TO
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

(B)


[PteGlu$_3$] (μM)$^{-1}$
0.8
1
2
4
8
10
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
-0.3
-0.2
-0.1
0.0
0.0
TOMATO JUICE (%, w/v)

(C)

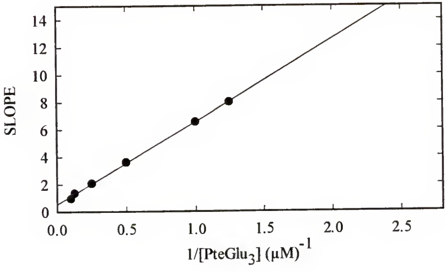
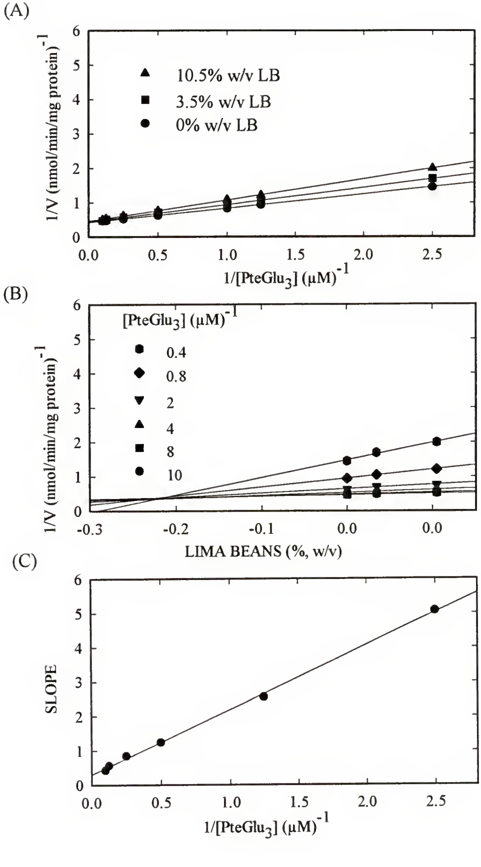

SLOPE
0
2
4
6
8
10
12
14
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

Figure 24. Inhibitory effect of lima beans on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.


(A)
1/V (nmol/min/mg protein)$^{-1}$
10.5% w/v LB
3.5% w/v LB
0% w/v LB
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$
(B)
1/V (nmol/min/mg protein)$^{-1}$
[PteGlu$_3$] (μM)$^{-1}$
0.4
0.8
2
4
8
10
0
1
2
3
4
5
6
-0.3
-0.2
-0.1
0.0
0.0
0.0
LIMA BEANS (%, w/v)
(C)
SLOPE
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

(K_i=6.48 mM) (Figure 25~28). These results are supported by Lineweaver-Burk plot, Dixon plot, and Dixon replot.

Reversibility of Inhibitory Effect on Porcine PPH Activity

The inhibition of porcine PPH activity by selected food extracts including orange juice, tomato juice, and lima beans was not reversed by increasing the concentration of zinc acetate (100~700 μM) in the reaction (Figure 29). These results suggested that the inhibition of porcine PPH activity was not mediated by chelating zinc ions.

Part II: Effects of Selected Food Components on Folate Bioavailability in Humans

Serum and Erythrocyte Folate

Serum and erythrocyte folate concentrations of human subjects in the first-term, mid-term, and final-term are shown in Tables 11 and 12. The folate concentrations in serum of the human subjects, as expected in this protocol, are much higher than the minimal range (≥6.0 ng/mL for serum, ≥160 ng/mL for erythrocyte; Sauberlich, 1977) and stay consistent during the six trials. Similarly, erythrocyte values were high, but increased over the course of the study ($P < 0.05$).

Urinary Folate

Typical chromatograms and spectra of folates and *p*ABG in standards and urine samples are shown in Figures 30 and 31. Effects of selected food extracts and 52 mM citric acid on folate excretion in urine of human subjects during the trials are shown in

Figure 25. Inhibitory effect of ascorbic acid on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.

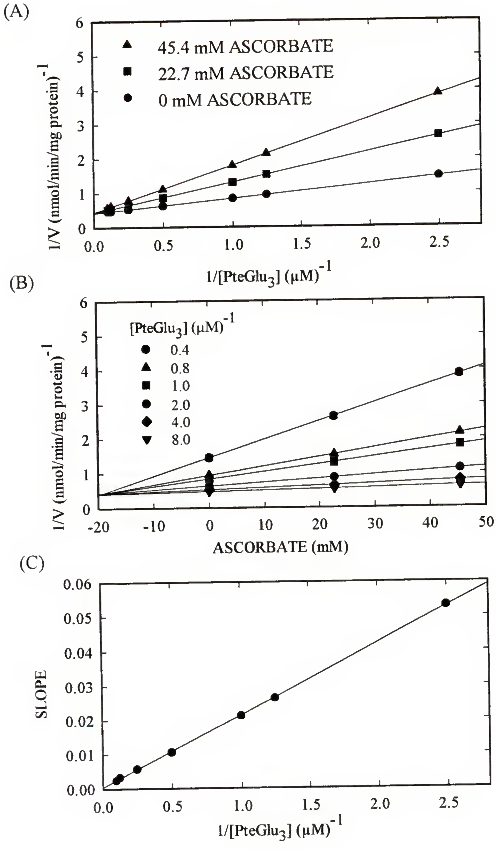

(A)
45.4 mM ASCORBATE
22.7 mM ASCORBATE
0 mM ASCORBATE
$1/V$ (nmol/min/mg protein)$^{-1}$
$1/[PteGlu_3]$ (μM)$^{-1}$
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
(B)
$[PteGlu_3]$ (μM)$^{-1}$
0.4
0.8
1.0
2.0
4.0
8.0
$1/V$ (nmol/min/mg protein)$^{-1}$
ASCORBATE (mM)
-20
-10
0
10
20
30
40
50
(C)
SLOPE
0.00
0.01
0.02
0.03
0.04
0.05
0.06
$1/[PteGlu_3]$ (μM)$^{-1}$

Figure 26. Inhibitory effect of citric acid on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.

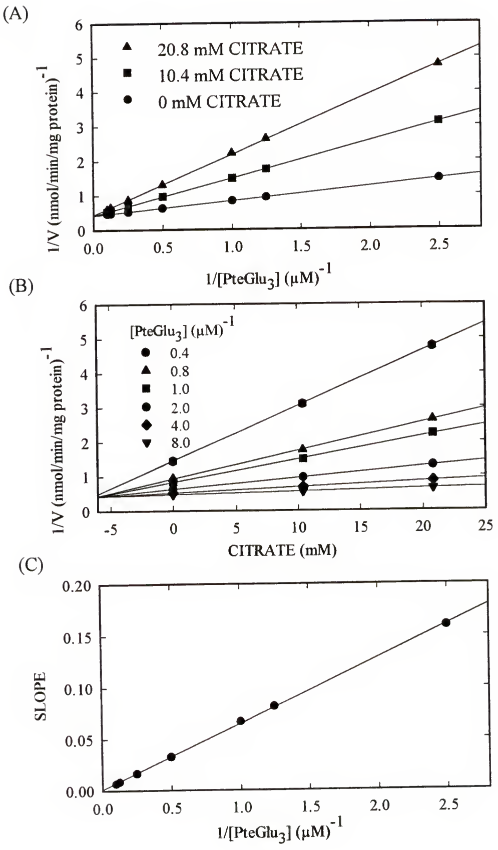

(A)
20.8 mM CITRATE
10.4 mM CITRATE
0 mM CITRATE
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$
(B)
[PteGlu$_3$] (μM)$^{-1}$
0.4
0.8
1.0
2.0
4.0
8.0
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
-5
0
5
10
15
20
25
CITRATE (mM)
(C)
SLOPE
0.00
0.05
0.10
0.15
0.20
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

Figure 27. Inhibitory effect of malic acid on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.

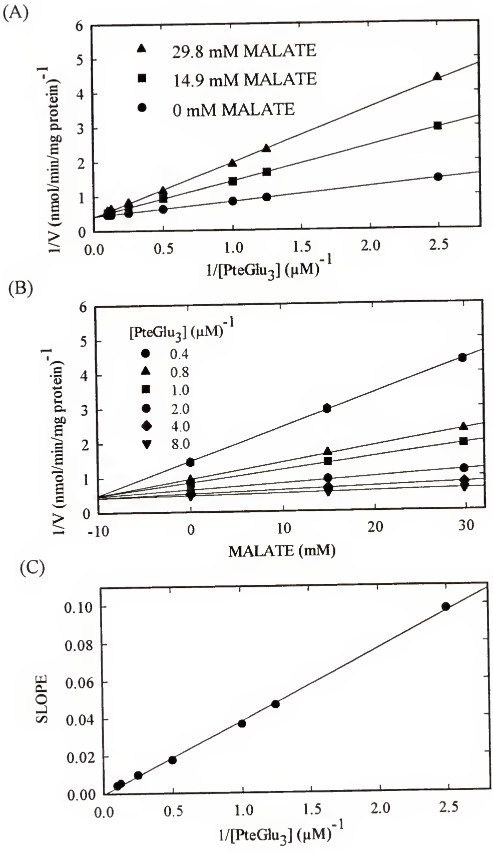
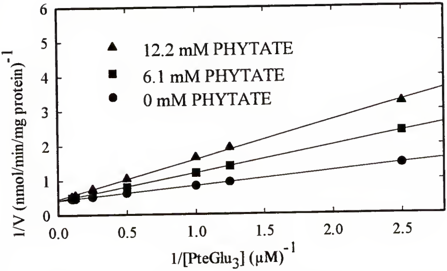
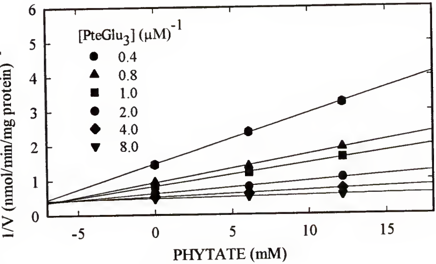

(A)
29.8 mM MALATE
14.9 mM MALATE
0 mM MALATE
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$
(B)
[PteGlu$_3$] (μM)$^{-1}$
0.4
0.8
1.0
2.0
4.0
8.0
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
-10
0
10
20
30
MALATE (mM)
(C)
SLOPE
0.00
0.02
0.04
0.06
0.08
0.10
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

Figure 28. Inhibitory effect of phytic acid on porcine PPH activity: (A) Lineweaver-Burk plot, (B) Dixon plot, (C) Dixon replot.

(A)


12.2 mM PHYTATE
6.1 mM PHYTATE
0 mM PHYTATE
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

(B)


[PteGlu$_3$] (μM)$^{-1}$
0.4
0.8
1.0
2.0
4.0
8.0
1/V (nmol/min/mg protein)$^{-1}$
0
1
2
3
4
5
6
-5
0
5
10
15
PHYTATE (mM)

(C)

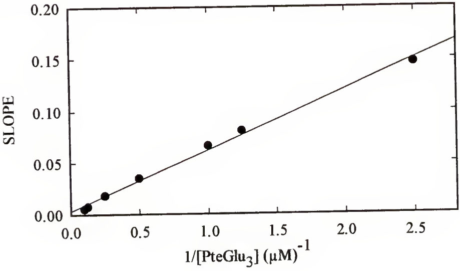

SLOPE
0.00
0.05
0.10
0.15
0.20
0.0
0.5
1.0
1.5
2.0
2.5
1/[PteGlu$_3$] (μM)$^{-1}$

Figure 29. Effects of zinc on porcine PPH activity in the presence of selected food extracts: (A) orange juice, (B) tomato juice, and (C) lima beans.

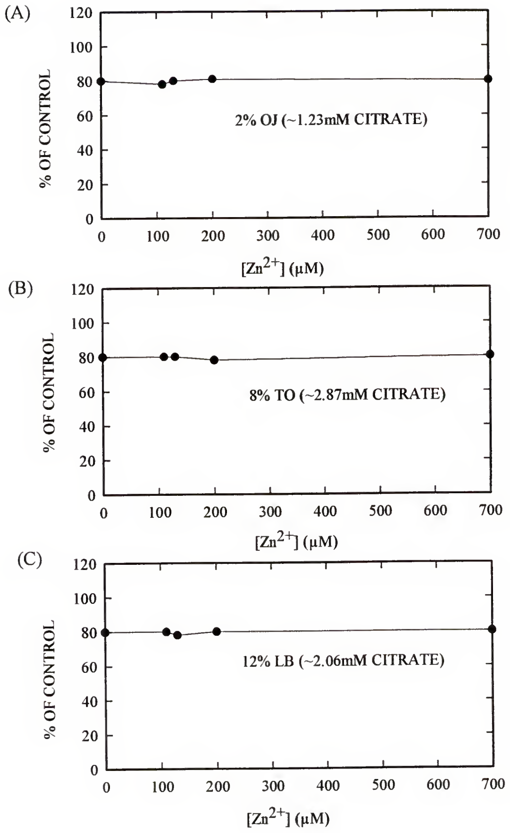

(A)
% OF CONTROL
0
20
40
60
80
100
120
0
100
200
300
400
500
600
700
[Zn^{2+}] (μM)
2% OJ (~1.23mM CITRATE)
(B)
% OF CONTROL
0
20
40
60
80
100
120
0
100
200
300
400
500
600
700
[Zn^{2+}] (μM)
8% TO (~2.87mM CITRATE)
(C)
% OF CONTROL
0
20
40
60
80
100
120
0
100
200
300
400
500
600
700
[Zn^{2+}] (μM)
12% LB (~2.06mM CITRATE)

Table 11. Serum folate concentrations (ng/mL) of human subjects in the trial 1 (first-term), trial 4 (mid-term), and trial 6 (final-terms)[1]

	First-term[a]	Mid-term[a]	Final-term[a]
Subject 1	28.8 ± 3.3	26.9 ± 2.5	21.5 ± 1.1
Subject 2	37.1 ± 1.5	37.8 ± 0.2	35.9 ± 5.4
Subject 3	32.8 ± 2.2	30.0 ± 2.4	21.0 ± 4.0
Subject 4	17.0 ± 1.9	14.9 ± 2.9	21.8 ± 2.8
Subject 5	30.8 ± 2.1	19.6 ± 1.7	25.7 ± 2.4
Subject 6	32.8 ± 2.9	30.2 ± 1.0	27.4 ± 4.3
Subject 7	-[2]	24.6 ± 2.3	20.6 ± 0.4

[1] Values are mean±SD (n=3).

[2] Sample was lost.

[a] Mean values for each term were not significantly different, as determined by one way repeated measures ANOVA (*P* =0.403).

Table 12. Red blood cell folate concentrations (ng/mL) of human subjects in the trial 1 (first-term), trial 4 (mid-term), and trial 6 (final-terms)[1]

	First-term[a]	Mid-term[a]	Final-term[b]
Subject 1	406 ± 18	399 ± 58	476 ± 44
Subject 2	313 ± 20	296 ± 24	497 ± 39
Subject 3	453 ± 47	407 ± 29	496 ± 20
Subject 4	209 ± 29	273 ± 17	382 ± 45
Subject 5	328 ± 7	358 ± 9	505 ± 53
Subject 6	225 ± 23	320 ± 26	460 ± 17
Subject 7	-[2]	362 ± 37	467 ± 59

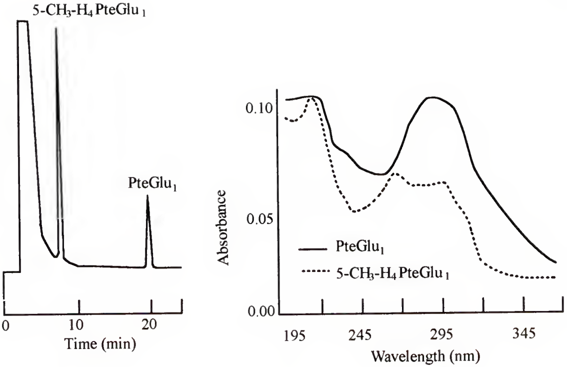
[1] Values are mean±SD (n=3).

[2] Sample was lost.

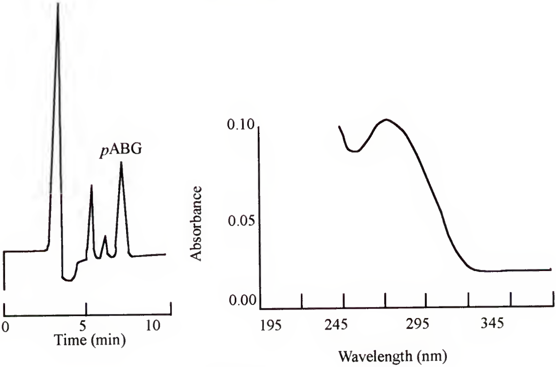
[a] Mean values for first-term and mid-term were not significantly different, as determined by one way repeated measures ANOVA (*P* =0.403).

[b] Mean values for first-term and mid-term were significantly different from final-term, as determined by one way repeated measures ANOVA (*P* <0.05).

Figure 30. Typical chromatograms and spectra of standard: (A) folates and (B) *p*ABG.

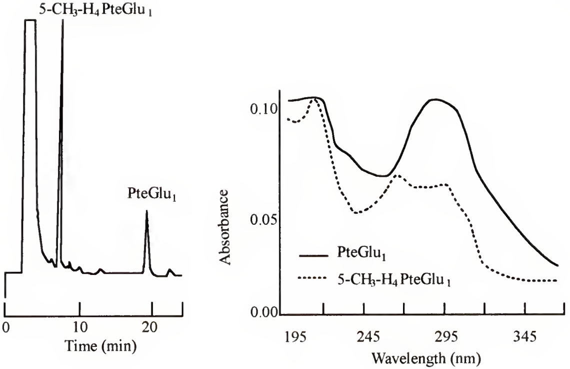


(A) folates

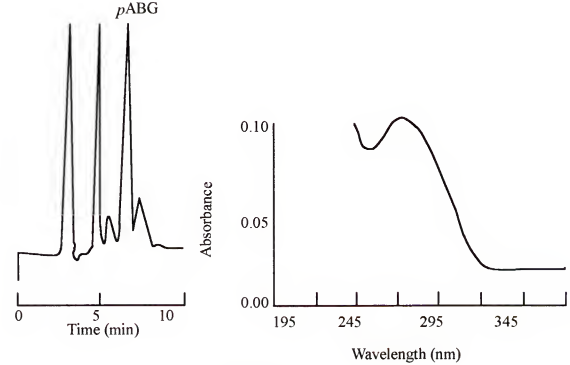


(B) *p*ABG

Figure 31. Typical chromatograms and spectra of: (A) folates and (B) *p*ABG in human urine.



(A) folates



(B) *p*ABG

Table 13. The urinary folate excretion in human subjects during the six trials averaged approximately 1 μmol/d. Only a few excretions were below 1 μmol/d and these could not be explained. Evidence gathered in recent stable isotopic studies in this laboratory and mass spectral results in this study indicated an apparent partial loss of a deuterium atom from d_4-folate in the course of GC/MS analysis. This may have been due to acid-catalyzed exchange during derivatization. To compensate for this process, mass spectral calculations for d_4-folate included the GC/MS signals obtained for both d_3-*p*ABG and d_4-*p*ABG lactams. Figure 32 illustrates a typical GC/MS chromatogram of derivatised d_0, d_2, d_3, and d_4 *p*ABG lactam derivatives. The d_2/d_0, d_3/d_0, and d_4/d_0 data from GC/MS for subject 1 in trial 1 are shown in Table 14. The data of day 1 (0~24 h predose period) reflected very little labeled folates, therefore the data of day 1 are not shown in Tables 15~20. The data for day 2 (0~24 h predose period) and day 3 (0~24 h postdose period) were considered respectively and continuously (0~24 h postdose period). Table 15 shows urinary excretion of d_2-folate by human subjects during the trials. Urinary excretion of d_3- plus d_4-folate by human subjects during the trials are shown in Table 16.

The percentage recovery of d_2-PteGlu$_6$ dose as urinary d_2-folate for human subjects during the trials is shown in Table 17 and Figure 33. Statistical differences in different trials were evaluated by one way repeated measures ANOVA. The data were subjected to a square-root transformation prior to this analysis to assure a normal distribution. The power of the performed test (0.773) was below the desired power of 0.8 which means that the interpretation of the negative findings should be very cautiously. The differences in the mean values among the trials are greater than would be expected by chance; there is

Table 13. Effects of selected foods and citric acid on total folate excretion (μmol/d) in urine of human subjects during the trials

Subject	Day[1]	Control[2]	Orange juice[3]	Tomato juice[3]	Lima beans[3]	Citric acid[3]	Control[2]
1	1	1.16	1.40	2.61	2.69	2.24	1.73
	2	0.51	0.55	1.44	0.39	2.07	0.19
	3	1.32	1.04	1.77	1.26	1.33	0.027
2	1	1.50	0.62	0.77	0.97	0.87	1.47
	2	1.44	1.25	2.03	0.94	1.93	0.83
	3	1.88	1.36	2.06	0.80	0.33	0.026
3	1	1.95	0.90	2.32	2.15	2.41	0.90
	2	2.10	0.52	0.73	0.90	1.33	0.85
	3	0.82	1.92	3.24	1.93	2.17	0.013
4	1	2.06	1.42	3.01	2.32	2.80	2.55
	2	1.07	1.56	1.80	1.76	3.68	0.67
	3	2.06	2.42	2.45	2.06	2.41	0.426
5	1	1.29	1.37	1.14	1.23	2.70	0.75
	2	0.98	0.42	0.37	1.19	2.00	0.60
	3	1.14	1.27	1.49	0.89	2.14	0.131
6	1	2.18	0.00	3.20	3.48	8.61	1.75
	2	2.55	0.70	2.54	3.47	4.93	2.51
	3	2.40	2.30	1.98	2.44	1.75	3.84
7	1	1.81	1.52	1.70	2.36	3.60	2.78
	2	2.07	1.39	2.10	2.31	4.23	0.49
	3	1.59	2.09	3.44	1.70	3.75	0.064

[1] Day 1 represents 0~24 h predose, day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose.

[2] All subjects were given water for control trials.

[3] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

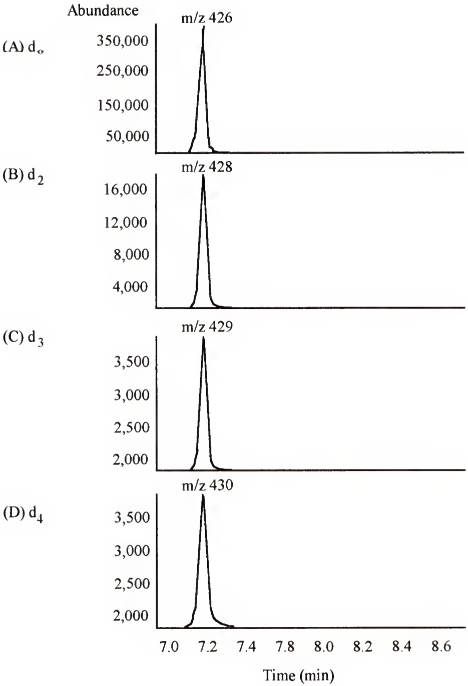


Figure 32. Typical GC/MS chromatograms of derivatised *p*ABG lactams: (A) d_0, (B) d_2, (C) d_3, and (D) d_4.

Table 14. Measured molar ratios of urinary folates excreted by Subject one in trial 1[1, 2] before and after administration of d_2-PteGlu$_6$ and d_4-PteGlu$_1$.

	Day 1 0~24 h predose	Day 2 0~24 h postdose	Day 3 24~48 h postdose
d_2 / d_0 (%)	0.388 ± 0.0214	3.33 ± 0.156	3.23 ± 0.0140
d_3 / d_0 (%)	0.015 ± 0.0016	1.51 ± 0.140	0.238 ± 0.0149
d_4 / d_0 (%)	0.139 ± 0.0036	2.36 ± 0.318	0.815 ± 0.0375

[1] In trial one subject was given water for control trial.

[2] Values are mean±SD (n=3).

Table 15. Urinary excretion (nmol/d) of d_2-folate by human subjects during the trials

Subject	Day[1]	Control[2]	Orange juice[3]	Tomato juice[3]	Lima beans[3]	Citric acid[3]	Control[2]
1	2	16.8	16.5	54.1	5.4	63.7	62.4
	3	15.9	5.8	25.4	9.1	10.1	0.3
	2+3	32.7	22.3	79.5	14.5	73.8	62.7
2	2	64.1	30.1	92.3	36.1	92.1	51.8
	3	44.8	39.0	40.3	9.1	4.8	1.5
	2+3	113	69.1	133	45.2	96.9	53.3
3	2	59.3	43.0	26.9	20.0	47.6	39.0
	3	17.0	28.4	37.3	13.0	18.6	0.3
	2+3	76.3	71.4	64.2	33.0	66.2	39.3
4	2	48.9	46.0	65.7	60.5	123	25.9
	3	38.0	0.2	28.7	39.1	20.0	1.3
	2+3	86.9	46.2	94.4	99.6	143	27.2
5	2	15.4	15.1	16.4	33.4	48.0	27.7
	3	16.2	9.0	14.5	5.4	29.7	21.1
	2+3	31.6	24.1	30.9	38.8	77.7	48.8
6	2	49.8	24.6	72.0	42.8	103.2	93.6
	3	38.5	8.7	37.8	5.8	12.8	60.8
	2+3	88.3	33.3	110	48.6	116.0	154
7	2	74.3	24.0	88.8	62.5	18.4	25.9
	3	30.3	7.9	17.9	12.0	20.6	4.6
	2+3	105	31.9	96.7	74.5	39.0	30.5

[1] Day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose, day 2+3 represents 0~48 h postdose.

[2] All subjects were given water for control trials.

[3] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

Table 16. Urinary excretion (nmol/d) of d_3- plus d_4-folates by human subjects during the trials

Subject	Day[1]	Control[2]	Orange juice[3]	Tomato juice[3]	Lima beans[3]	Citric acid[3]	Control[2]
1	2	17.1	21.1	55.1	6.1	83.4	78.3
	3	14.9	14.6	26.7	12.2	15.0	0.4
	2+3	32.0	35.7	81.8	18.3	98.4	78.7
2	2	56.3	70.4	104	39.6	106	53.9
	3	24.2	75.7	25.1	17.3	8.1	1.0
	2+3	80.5	146	129	56.9	114	54.9
3	2	32.1	35.6	24.6	7.3	55.8	34.0
	3	14.1	25.0	51.7	10.1	31.1	0.3
	2+3	46.2	60.6	76.3	17.4	86.9	34.3
4	2	48.2	81.3	67.7	74.3	163	32.2
	3	27.7	58.3	41.7	23.3	31.8	25.6
	2+3	75.9	140	109	97.6	194	57.8
5	2	25.0	26.9	53.4	24.7	61.6	29.8
	3	11.2	22.8	22.7	9.1	29.3	13.5
	2+3	36.2	49.7	76.1	33.8	90.9	43.3
6	2	66.3	28.0	118	106	113	97.8
	3	41.7	32.2	27.8	20.2	25.8	99.0
	2+3	108	60.2	145	126	139	187
7	2	72.6	78.9	101	2.7	45.0	31.7
	3	21.6	35.7	45.8	2.5	43.4	1.6
	2+3	94.2	114	146	5.2	88.4	33.3

[1] Day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose, day 2+3 represents 0~48 h postdose.

[2] All subjects were given water for control trials.

[3] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

Table 17. Percentage recovery of d_2-PteGlu$_6$ dose as urine d_2-folate by human subjects during the trials

Subject	Day[1]	Control[2]	Orange juice[3]	Tomato juice[3]	Lima beans[3]	Citric acid[3]	Control[2]
1	2	3.35	3.29	10.0	1.09	12.7	12.4
	3	3.17	1.15	5.07	1.81	1.98	0.154
	2+3	6.52	4.44	15.1	2.90	14.7	12.6
2	2	12.8	6.00	18.4	7.19	18.2	10.3
	3	7.01	7.77	8.04	1.89	0.958	0.10
	2+3	19.8	13.8	26.4	9.08	19.2	10.4
3	2	7.03	6.92	4.86	3.99	9.48	7.74
	3	3.40	5.65	7.43	2.58	3.72	0.173
	2+3	10.4	12.6	12.3	6.57	13.2	7.91
4	2	9.75	9.17	12.7	12.1	24.5	5.16
	3	6.29	4.58	5.72	7.80	3.96	0.252
	2+3	16.0	13.8	18.4	19.9	28.5	5.41
5	2	3.08	3.01	3.81	6.66	9.57	5.53
	3	3.23	1.85	2.90	1.08	5.93	1.76
	2+3	6.31	4.86	6.71	7.74	15.5	7.29
6	2	9.92	4.90	15.1	8.53	20.6	18.8
	3	7.68	1.74	7.54	1.16	2.56	15.6
	2+3	17.6	6.64	22.6	9.69	23.2	34.4
7	2	14.8	4.79	17.1	1.31	3.68	5.16
	3	6.03	1.58	3.56	2.44	4.11	1.01
	2+3	20.8	6.37	20.7	3.75	7.79	6.17

[1] Day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose, day 2+3 represents 0~48 h postdose.

[2] All subjects were given water for control trials.

[3] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

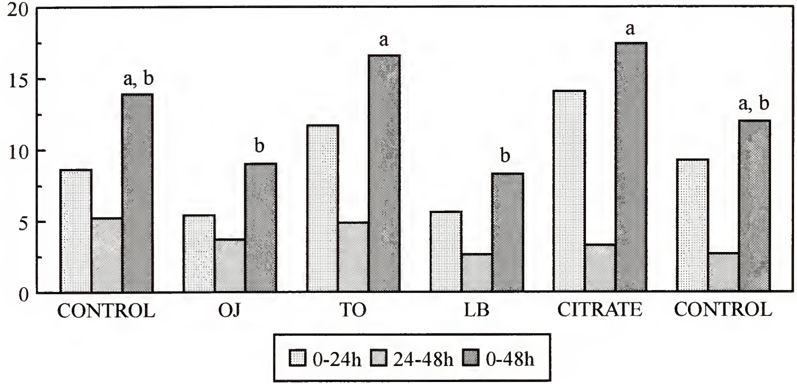


Figure 33. Percentage recovery of d_2 -PteGlu$_6$ dose as urine d_2-folate by human subjects during the trials.[1]

[1] Values with the same superscript letter were not significantly different ($P > 0.05$). Statistical analysis was only performed on 0~48 h data.

a significant difference (*P*=0.00781). Multiple comparisons to isolate the trial or trials that differ from the others were performed using the Student-Newman-Keuls method. The results indicate that there are significantly differences between citric acid trial vs lima bean trial; citric acid trial vs orange juice trial; tomato juice trial vs lima bean trial; tomato juice vs orange juice trial, and there are no significantly differences between the control trial vs the tested food trials.

Table 18 and Figure 34 show the percentage recovery of d_4-PteGlu$_1$ dose as urinary d_3- plus d_4-folate for human subjects during the trials. Statistical differences in different trials were evaluated by one way repeated measures ANOVA. The power of the performed test (0.64) was below the desired power of 0.8 which again means that the interpretation of the negative findings should be very cautiously. The differences in the mean values among the trials were greater than would be expected by chance; there is a significant difference (*P*=0.0188). Multiple comparisons were again performed by Student-Newman-Keuls method. The results indicate that there were significantly differences between the citric acid trial vs lima bean trial and there were no significantly differences between the control trial vs the tested food trials.

The percentage recovery of d_2-PteGlu$_6$ dose as urinary d_2-folate and the percentage recovery of d_4-PteGlu$_1$ dose as urinary d_3- plus d_4-folate for human subjects during the trials may be different for samples collected at different times. To minimize between-subject variation in folate excretion characteristics and to increase the power of the bioavailability protocol, the absorption of the polyglutamate and monoglutamate was directly compared by using the excretion ratio. For each subject in an individual trial, the

Table 18. Percentage recovery of d_4-PteGlu$_1$ dose as urine d_3- plus d_4-folates by human subjects during the trials

Subject	Day[1]	Control[2]	Orange juice[3]	Tomato juice[3]	Lima beans[3]	Citric acid[3]	Control[2]
1	2	2.95	3.65	10.1	1.05	14.4	13.5
	3	2.73	2.52	4.62	2.10	2.59	0.172
	2+3	5.68	6.17	14.7	3.15	17.0	13.7
2	2	10.3	12.2	18.0	6.85	18.2	9.33
	3	3.85	13.7	4.35	2.98	1.40	0.11
	2+3	14.2	25.9	22.4	9.73	19.6	9.44
3	2	5.76	6.16	4.27	3.25	9.64	5.89
	3	2.44	4.32	8.93	1.74	5.37	0.191
	2+3	8.20	10.5	13.2	6.24	15.0	6.08
4	2	8.32	14.1	12.5	12.8	28.1	5.57
	3	4.78	12.6	7.20	4.03	5.50	0.232
	2+3	13.1	26.7	19.7	16.8	33.6	5.80
5	2	4.32	4.65	4.22	4.26	10.6	5.15
	3	1.93	3.93	3.93	1.58	5.16	1.40
	2+3	6.25	8.58	8.15	5.84	15.8	6.55
6	2	11.5	4.84	20.3	17.6	19.6	16.9
	3	7.21	3.56	4.81	3.49	4.42	14.7
	2+3	18.7	8.40	25.1	21.1	24.0	31.6
7	2	17.6	13.6	17.4	1.72	7.77	5.48
	3	3.73	6.16	7.92	2.35	7.50	1.28
	2+3	21.3	19.8	25.3	4.07	15.3	6.76

[1] Day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose, day 2+3 represents 0~48 h postdose.

[2] All subjects were given water for control trials.

[3] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

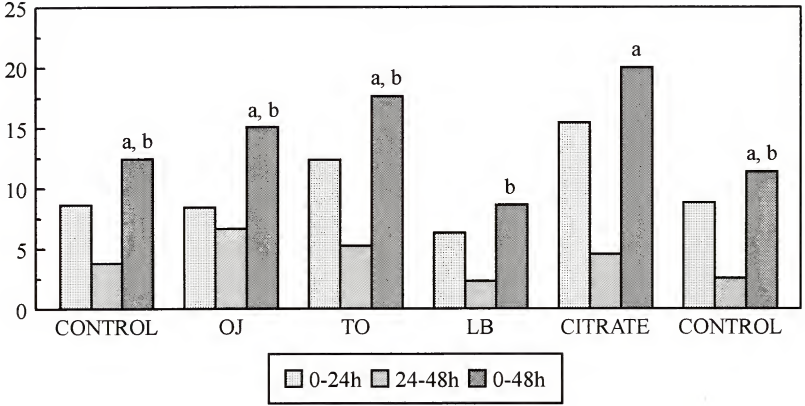


Figure 34. Percentage recovery of d_4 -PteGlu$_1$ dose as urine d_3- plus d_4 -folates by human subjects during the trials.[1]

[1] Values with the same superscript letter were not significantly different ($P > 0.05$). Statistical analysis was only performed on 0~48 h data.

urinary excretion ratio of pteroylpolyglutamate vs pteroylmonoglutamate was calculated as shown below:

$$\text{excretion ratio} = \frac{\%\text{ of } d_2\text{-PteGlu}_6 \text{ dose excreted as } d_2\text{-folates}}{\%\text{ of } d_4\text{-PteGlu}_1 \text{ dose excreted as } d_3\text{- plus } d_4\text{-folates}}$$

Table 19 and Figure 35 show the urinary excretion ratio (as defined above) of the human subjects during the trials. A ratio of 1.0 would indicate equivalent absorption and excretion of d_2-PteGlu$_6$ and d_4-PteGlu$_1$. A ratio lower than one would indicate less complete absorption of the polyglutamyl folate. Statistical differences in different trials were evaluated by one way repeated measures ANOVA. The power of the performed test (0.917) was above the desired power of 0.8. The differences in the mean values among the trials were greater than would be expected by chance; there was a significant difference (P=0.00169). To isolate the trial or trials that differ from the others, the Student-Newman-Keuls method was used for multiple comparisons. The results indicate that there were significant differences between the control trial vs orange juice trial; citric acid trial vs orange juice trial; tomato juice trial vs orange juice trial; lima bean trial vs orange juice trial, and there were no significant differences between the two control trials. The 48 h ratio of urinary d_2/d_4-folates indicated lower bioavailability of the d_2-PteGlu$_6$ in the orange juice trial (P <0.05; ~66% relative to that in the control trials), while other treatments did not differ significantly from the two control trials. Table 20 lists the mean values and standard deviation for all subjects of total urinary folate, labeled urinary folates, percentage recovery of labeled folates, and urinary excretion ratio of pteroylpolyglutamate vs pteroylmonoglutamate. In the control (trial 1) the $d_2/(d_3+d_4)$

Table 19. Urinary excretion ratio of pteroylpolyglutamate vs pteroylmonoglutamate by human subjects during the trials

Subject	Day[1]	Control[2]	Orange juice[3]	Tomato juice[3]	Lima beans[3]	Citric acid[3]	Control[2]
1	2	1.14	0.90	0.99	1.04	0.88	0.92
	3	1.16	0.46	1.10	0.86	0.76	0.90
	2~3	1.15	0.72	1.03	0.92	0.86	0.92
2	2	1.24	0.49	1.02	1.05	1.00	1.10
	3	1.82	0.57	1.85	0.63	0.68	0.88
	2~3	1.40	0.53	1.18	0.93	0.98	1.10
3	2	1.22	1.12	1.14	1.23	0.98	1.31
	3	1.39	1.31	0.83	1.48	0.69	0.91
	2~3	1.27	1.20	0.93	1.05	0.88	1.30
4	2	1.17	0.65	1.02	0.94	0.87	0.92
	3	1.31	0.36	0.79	1.94	0.72	1.09
	2~3	1.22	0.52	0.94	1.18	0.85	0.93
5	2	0.71	0.65	0.90	1.56	0.90	1.07
	3	1.67	0.47	0.74	0.68	1.15	1.26
	2~3	1.01	0.57	0.82	1.33	0.98	1.11
6	2	0.87	1.01	0.74	0.49	1.05	1.11
	3	1.07	0.49	1.57	0.33	0.58	1.06
	2~3	0.94	0.79	0.90	0.46	0.96	1.09
7	2	0.84	0.35	0.98	0.76	0.47	0.94
	3	1.62	0.26	0.45	1.04	0.55	0.79
	2~3	0.98	0.32	0.82	0.92	0.51	0.91

[1] Day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose, day 2+3 represents 0~48 h postdose.

[2] All subjects were given water for control trials.

[3] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

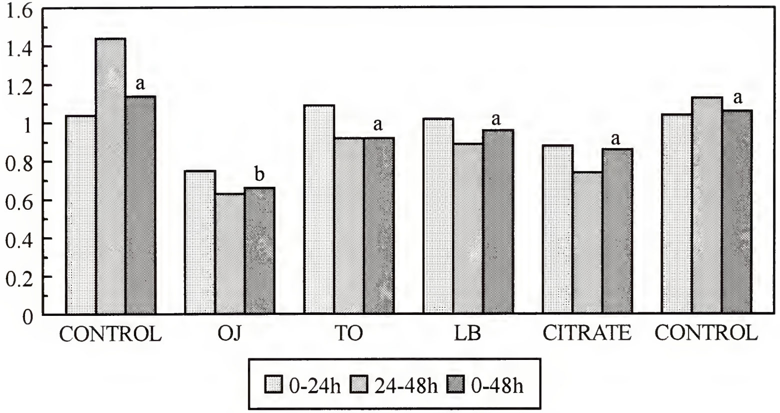


Figure 35. Urinary excretion ratio of pteroylpolyglutamate vs pteroylmonoglutamate by human subjects during the trials.[1]

[1] Values with the same superscript letter were not significantly different ($P > 0.05$). Statistical analysis was only performed on 0~48 h data.

Table 20. Urinary excretion of total deuterium-labeled folates by human subjects during the trials[1]

	Day[2]	Control[3]	Orange juice[4]	Tomato juice[4]	Lima beans[4]	Citric acid[4]	Control[3]
Total urinary folates (µmol)	2	1.53±0.73	0.91±0.47	1.57±0.78	1.57±1.06	2.88±1.38	0.88±0.76
	3	1.60±0.55	1.77±0.55	2.35±0.74	1.58±0.62	1.98±1.05	0.65±1.42
	2+3	3.13±1.03	2.68±0.88	3.92±1.15	3.15±1.54	4.86±2.08	1.52±2.15
Labeled urinary folates (nmol)							
d_2	2	46.9±22.8	28.5±12.1	59.5±29.1	37.2±20.6	70.9±36.7	46.6±25.0
	3	28.7±12.3	14.1±14.0	28.8±10.2	13.4±11.7	16.7±8.2	12.8±22.4
	2+3	76.3±32.5	42.6±20.4	87.0±33.0	50.6±28.2	87.5±34.3	59.4±43.6
d_3+d_4	2	45.4±21.2	48.9±26.7	74.8±33.7	37.2±39.4	89.7±41.1	51.1±27.1
	3	22.2±10.4	37.8±21.7	34.5±11.6	13.5±7.18	26.4±11.6	20.2±36.0
	2+3	67.6±29.7	86.6±45.6	109±31.5	50.7±45.5	116±39.1	69.9±54.0
% recovery of folates[5]							
d_2	2	8.68±4.46	5.44±2.15	11.7±5.75	5.84±3.99	14.1±7.30	9.30±5.03
	3	5.26±1.94	3.47±2.55	5.75±2.03	2.68±2.33	3.32±1.63	2.72±5.71
	2+3	13.9±6.12^{a}	8.93±4.27^{b}	17.5±6.65^{a}	8.52±5.63^{b}	17.4±6.84^{a}	12.0±10.2^{a}
d_3+d_4	2	8.68±5.01	8.46±4.62	12.4±6.55	6.79±6.20	15.5±7.10	8.83±4.68
	3	3.81±1.78	6.68±4.56	5.97±2.00	2.61±0.92	4.56±2.02	2.58±5.37
	2+3	12.5±6.10^{ab}	15.2±8.77^{ab}	18.4±6.53^{ab}	9.56±6.85^{b}	20.0±6.77^{a}	11.4±9.33^{ab}
$d_2/(d_3+d_4)$ ratios[5]	2	1.03±0.21	0.74±0.28	0.97±0.12	1.01±0.34	0.88±0.19	1.05±0.14
	3	1.43±0.28	0.56±0.35	1.05±0.50	0.99±0.55	0.73±0.20	0.98±0.16
	2~3	1.14±0.17^{a}	0.66±0.28^{b}	0.95±0.13^{a}	0.97±0.27^{a}	0.86±0.16^{a}	1.05±0.14^{a}

[1] Values are mean±SD (n=3).

[2] Day 2 represents 0~24 h postdose, day 3 represents 24~48 h postdose, day 2+3 represents 0~48 h postdose.

[3] All subjects were given water for control trials.

[4] Subjects were given orange juice, tomato juice, lima beans, and 52 mM citric acid, respectively in trial 2~5.

Table 20.-- Continued.

[5] Values with the same superscript letter were not significantly different ($P > 0.05$). Statistical analysis was only performed on 0~48 h d_2 and d_3 plus d_4 percentage recovery of folates and $d_2/(d_3+d_4)$ excretion ratios.

excretion ratio was 1.14±0.17 (Mean±SD). This indicates that d_2-PteGlu$_6$ and d_4-PteGlu$_1$ exhibited similar absorption and excretion patterns. The observation of no significant difference between the two controls (trial 1: 1.14±0.17 vs trial 6: 1.05±0.17) indicates that there were no changes during this long study period. The $d_2/(d_3+d_4)$ excretion ratio in the orange juice trial, as reported previously, was lower than control (0.66±0.28), which reflected a lower rate of absorption of d_2-PteGlu$_6$ relative to d_4-PteGlu$_1$ ($P < 0.05$). The ratios in the tomato juice trial (0.95±0.15), lima beans trial (0.97±0.27), and citric acid trial (0.86±0.16) were close to the two control trials. These results suggest that orange juice exhibited incomplete polyglutamate bioavailability in humans under the conditions of this trial. However, the power of this trial did not permit a complete assessment of the significance of any effect of tomatoes and beans on folate bioavailability.

DISCUSSION

Factors including foods, drugs, alcohol, stress, and seasonal adaptations could all have an influence on bioavailability. A definition of bioavailability should include digestion, absorption, post-absorptive functions, metabolism, transport, conversion of metabolically inactive forms to active forms, incorporation into tissue, and finally urinary and fecal excretion. In the context of this study, digestion and absorption variables are the major factors affecting bioavailability. Thus folate bioavailability can be affected by a variety of factors including impairment of intraluminal deconjugation, binding, entrapment, or by impairment of the transport process. To assess folate requirements and bioavailability of naturally occurring dietary folate, Sauberlich et al. (1987) conducted a 92-d controlled metabolic study in women. The subjects received various amounts of folate from dietary sources, added folic acid, or a combination of dietary and added folate. The investigators concluded that a daily intake of 300 μg of total dietary folate was sufficient to meet the folate requirement, which was substantially higher than the intake of folic acid found to meet the requirement. In comparison to the synthetic folic acid, the response to dietary folate indicated no more than 50% bioavailability. At present this is the most conclusive estimate of the overall bioavailability of folate from food sources.

The main objective of this study was to determine whether food extracts could influence folate bioavailability by decreasing the intraluminal deconjugation of dietary pteroylpolyglutamates to the absorbable pteroylmonoglutamate and identify the inhibitor(s) in food extracts. This research included two parts, an in vitro study to investigate the effects of selected food components on porcine PPH activity and to identify the inhibitor(s) in food extract and an in vivo study is to investigate whether food extracts could influence folate bioavailability.

Part I: Effects of Selected Food Components on Porcine PPH Activity

Several studies (Day and Gregory, 1984; Wang et al., 1986; Reisenauer et al., 1986; Gregory et al., 1987) have indicated that the pig would be a good animal model for studies of digestion and absorption of pteroylpolyglutamates. Because the PPH from porcine jejunal brush border membrane has similar pH optimum, zinc ion requirements, $PteGlu_n$ affinities, and physiological function to human jejunal brush border PPH (Bhandari and Gregory, 1990), porcine PPH was used as a source of the enzyme for the in vitro studies described here. Other animal species, including the monkey, rat, and guinea pig, lack significant jejunal brush border membrane PPH activity (Hoffbrand and Peters, 1969; Day and Gregory, 1984; Elsenhans et al., 1984; Wang et al., 1985 and 1986). In this study, porcine jejunal brush border membrane PPH exhibited similar K_m values (1.02 ± 0.02 μM) to human K_m values (0.9 μM) (Reisenauer and Halsted, 1981; Chandler et al., 1986) and could completely hydrolyze $PteGlu_3$ to $PteGlu_1$ via an exohydrolytic process (Figure 7).

PPH activity was eliminated by treatment with EDTA and phenanthroline, which are capable of tightly chelating zinc (formation constant of Zn^{2+}-EDTA complex = 3 x

10^{16}; Schwarzenbach, 1957). Enzyme activity was then fully restored by adding zinc acetate (2~30 μM) to the ion assay mixture (15 μg treated porcine jejunal brush border membrane vesicle protein, 30 mM Tris-HCl buffer, pH 7, and 10 μM $PteGlu_3$ as substrate) (K_m=11.8±1.40 μM) (Figure 8). This result suggests that the enzyme may be partially inactivated in vivo by the low levels of chelators present in human diets. Zinc deficiency in human subjects has been shown to decrease the utilization of an oral dose of pteroylheptaglutamate but not pteroylmonoglutamate (Tamura et al., 1978), presumably because of the requirement of the PPH enzyme for zinc as a cofactor. Other salts, including NaCl (20~400 mM), KCl (20~400 mM), $MgCl_2$ (20~100 mM), or $CaCl_2$ (20~100 mM) were added to the ion assay mixture but did not restore the activity of EDTA-treated porcine PPH. The results indicate that Na^+, K^+, Mg^{2+}, and Ca^{2+} ions are not cofactors for porcine PPH, which confirms Chandler's findings (Chandler et al., 1986). In addition, over 300 mM NaCl inhibited porcine PPH activity when incubated with 100 μM zinc acetate in the ion assay mixture (data not shown). For this reason, Tris-base rather than NaOH was used to adjust the food extracts to pH 7 in this study. NaCl at 300 mM has little or no physiological relevance with respect to digestive enzymology.

Many food extracts were found to have in vitro inhibitory effects on the jejunal brush border membrane PPH activity. In the present study, tomato juice and lima beans caused a moderate inhibition of the enzyme activity and orange juice caused a strong inhibition (Figures 9, 10, 11). The observed in vitro inhibition of the jejunal brush border membrane PPH activity by certain food extracts, including orange juice, tomato juice, and lima beans, was not due to the pH because all the food extracts were neutralized before testing. In addition, the pH of the enzyme assay system in the presence of these food extracts at the beginning and at the end of the enzyme reaction was the same. The reaction was conducted at a physiologically relevant pH. Optimum pH for the activity of jejunal brush border membrane PPH activity is neutral to slightly acidic (Chandler et al., 1986; Wang et al., 1985) whereas the pH optimum for folate transport into intestinal mucosal cell is slightly acidic (Said et al., 1987).

The results of this study indicate that anionic components with molecular weight less than 6kD had an inhibitory effect on porcine PPH activity. Since orange juice and tomato juice are rich in citric acid and malic acid (Sinclair, 1961; Miladi et al., 1969; Gould, 1974) and lima beans contained high amounts of phytic acid (Oberleas and Harland, 1981), it is probable that organic acids, including citric acid, malic acid, and phytic acid, were largely responsible for this inhibition. By using the ion exchange HPLC chromatography, the organic acids in food extracts were separated. By spiking, these specific organic acid peaks could be confirmed with similar retention time comparing to standard. However, using A_{210}/A_{220} ratio to identify the organic acids showed that the ratio of organic acid peaks in food extracts were not all identical to the standard organic acids. This result indicates that these organic acid peaks were not totally pure (Table 9).

Ion chromatography with a conductivity detector was also used to quantify the inhibitors in food extracts. Table 10 lists that orange juice contained 61.4 mM of citric acid and 47.7 mM of malic acid. These values for both acids are higher than published data (Sinclair, 1961; citric acid = 52 mM and malic acid = 37.3 mM). Tomato juice was found to contain 35.9 mM citric acid and 16.4 mM of malic acid. Citric acid in tomato juice was also higher than published values (Miladi et al., 1969; Gould, 1974; citric acid = 26 mM and malic acid = 18.6 mM), while malic acid was close to published data. An interesting finding was that citric acid was found in lima beans, which was not expected. The possible explanation is that citric acid is adding as an acidulant as a processing aid. A high amount of phytic acid [116 mM; Oberleas and Harland, (1981) reported 152 mM phytic acid] was found in lima beans, but only a small amount was found in orange juice (1.83 mM) and tomato juice (3.71 mM). By using these organic acid concentrations, the percentage of inhibition due to these organic acids in food extracts could be estimated, as described later.

All three organic acids were derivatized for analysis by gas chromatography (GC), but only citric acid could be detected (Figure 18). Phytic acid could not dissolve in borontriflouride with methanol; therefore, phytic acid could not be derivatized and detected by GC. Malic acid can dissolve well but the result showed it has several peaks and cannot be repeated well. The method developed by Morrison and Smith (1964) was designed for fatty acids not for other organic acids. Due to the methodologic limitation, the gas chromatographic separation was only suitable for citric acid.

The effects of selected organic acids on porcine PPH activity indicate that the citric acid peak in tomato juice and orange juice had a marked inhibitory effect on the porcine jejunal brush border membrane PPH activity. Results in this study indicate that the citric acid peak strongly inhibited porcine PPH activity, while malic acid and phytic acid peaks showed a weak inhibitory effect on porcine PPH activity (Figures 16, 19~21). However, the citric acid in orange juice, tomato juice, and lima beans did not completely account for the observed inhibitory effect on the PPH activity. The inhibition was due to not only citric acid but also malic acid, phytic acid, and other compounds including ascorbic acid.

The mode of inhibition was evaluated by determining the reaction velocity with various concentrations of substrate $PteGlu_3$ in the presence and absence of neutralized food extracts. The Lineweaver-Burk plot, Dixon plot, and Dixon replots were used to identify the mode of inhibition by various food extracts. Inhibition of porcine PPH activity was competitive with respect to $PteGlu_3$ not only by selected food extracts including orange juice [K_i=2.14% (v/v)], tomato juice [K_i=5% (w/v)], and lima beans [K_i=14.7% (w/v)] (Figure 22, 23, and 24), but also by selected organic acids including ascorbic acid (K_i=19.6 mM), citric acid (K_i=6.42 mM), malic acid (K_i=10.1 mM), and phytic acid (K_i=6.48 mM) (Figures 25~28). Therefore the inhibition can be reversed by supplying more substrate $PteGlu_3$ in vitro. Based on these K_i values of organic acids and organic acids concentration in food extracts shown in Table 10, the percentage of inhibition of organic acids in food extracts can be estimated. However, the estimated percentage inhibition values are less than the experimental data. This finding indicates that there are not only three inhibitors (citric acid, malic acid, and phytic acid) in food extracts, a conclusion also reached in the chromatographic isolation of organic acids.

The reversibility of inhibition of porcine PPH activity by selected food extracts including orange juice, tomato juice, and lima beans was investigated by increasing zinc acetate from 0.1 mM to 0.7 mM in the reaction. However, it was not reversed by increasing the extra concentration of zinc acetate (100~700 μM) in the reaction (Figure

29). These results suggest that the inhibition of porcine PPH activity is not mediated by chelating zinc ions.

Rao and Noronha (1977) reported that citrate was the most potent inhibitor (100% inhibition at 0.4 M concentration) for chicken liver PPH. The potent inhibition by citrate ions at higher concentrations is suggestive of physiological mechanisms that could regulate the enzyme in vivo. Heparin was the most potent conjugase inhibitor producing 50% inhibition at a concentration 26.7 ng/mL for bovine hepatic PPH. High anion concentrations probably interfere with the polyanion nature of the polyglutamate substrate essential for its binding to the active site at the optimal pH (Silink et al., 1975).

To explain the low availability of folate from some plant-derived foods, it has been suggested that certain components may bind or entrap dietary folates (De Arends, 1979). However, Bhandari and Gregory (1990) reported that the recovery of $PteGlu_3$ determined by ultrafiltration was not affected by various food extracts (legumes, tomato juice, and orange juice). This indicates that soluble macromolecules in the food extracts did not bind $PteGlu_3$. Earlier studies from Gregory et al. (1987) indicated that soluble anionic polysaccharides, including polygalacturonic acid, pectin, alginic acid, and sulfated polydsaccharides did not significantly inhibit porcine jejunal brush border membrane PPH activity at concentrations as high as 1% (w/v) when in the presence of excess 100 μM zinc ions.

Part II: Effects of Selected Food Components on Folate Bioavailability in Humans

Many dietary and physiological factors can influence the deconjugation action of the PPH, thus affecting the digestion and absorption of the pteroylpolyglutamates (Bailey, 1988). Orange juice provides approximately 10% of total dietary intake of folate in the average American diet. Frozen orange juice reconstituted and appropriately diluted contains 125 µg/250 mL (Streiff, 1971). Folate in orange juice is stable because of the relatively high content of the antioxidant vitamin C. However, several studies have indicated that the bioavailability of folate in orange juice is potentially incomplete.

Rhode et al. (1983), using long-term feeding of orange juice as a folate supplement to women on folate-restricted diets, found that the folate in reconstituted orange juice did not differ significantly in bioavailability from synthetic folic acid. Tamura and Stokstad (1973) observed a low availability of folate in feeding large quantities of undiluted orange juice concentrate to folate saturated human subjects. In this same study orange juice at pH 3.7 was 35% bioavailable as compared to being 47% bioavailable at pH 5.1. The bioavailability of synthetic heptaglutamyl folate added to the orange juice was similar at both pH values (28% at pH 3.7 and 23% at pH 5.1). Tamura et al. (1976) indicated that 600 g of concentrated orange juice (pH 3.7) decreased the availability of 1 mg of supplemental PteGlu_7 to 54% as compared to control values (based on a standard curve). A synthetic orange juice solution was made to determine what the food component(s) of orange juice caused the inhibition. The bioavailability of PteGlu_7 was 66% and 39% when 12 and 24 g of citric acid was added to the synthetic orange juice solution, respectively. However, when the pH of the added citric acid was neutralized to a pH of

6.4, the $PteGlu_7$ bioavailability was 105%. These results suggest that excessively large quantities of orange juice cause an inhibition of the absorption of polyglutamyl folates. This effect is presumably a direct effect of the low pH on jejunal brush border membrane PPH activity. It should be noted that the studies by Tamura and Stokstad (1973) involved the use of large quantities of $PteGlu_7$ (1 mg/dose), which relative to the smaller doses used in this study, may have accentuated differences in bioavailability.

Abad and Gregory (1987) indicated that the apparent bioavailability of endogenous folate in dehydrated orange juice for rats was 146% relative to a folic acid standard in a dry casein-sucrose base diet. In contrast to the results for endogenous folate, the apparent bioavailability of added pteroylheptaglutamate was 101% in orange juice. In another study, Abad and Gregory (1988) observed in rats a slightly reduced (20-25%) bioavailability of added tritiated folates in gelled diets containing a nutritionally relevant concentration of orange juice solids. The result indicated that the bioavailability of both mono- and polyglutamate forms of administered [^{3}H]folate was slightly decreased due to orange juice ($P < 0.05$). This suggests that the impairment of in vivo retention was not due to the inhibition of PPH, and could be due to the foods binding to folate; decreased intestinal transit time; or an impaired absorption of the monoglutamates.

Clifford et al. (1990) determined the bioavailability of endogenous orange juice folate to be 62% using growth as a response criterion and 120% using liver folates as the response criteria in folate depleted rats. In a similar study the bioavailability of orange juice was determined to be as available as folic acid based on weight gain (98%), liver folate concentration (89%), total liver folate (88%), serum folate (98%), and erythrocyte folate (115%) (Clifford et al., 1991). The discrepancies between these two studies are not readily explained. It has been reported that the rat does not contain a brush border membrane PPH. Therefore, the use of the rat as a model for comparison to human absorption of polyglutamyl folates is not justified (Day and Gregory, 1984; Wang et al., 1985).

In general, the availability of folate from a wide variety of beans including lima beans is incomplete (Babu and Srikantia, 1976; Tamura and Stokstad, 1973; Devadas et al., 1979). However, Keagy et al. (1988) reported that California white beans had no effect on the bioavailability of exogenous pteroylpolyglutamate in human subjects. Incomplete folate bioavailability from tomatoes was also reported (Babu and Srikanita, 1976; Retief, 1969).

The reported interactions of zinc and folate have been investigated recently. With respect to the bioavailability of polyglutamates, human intestinal brush border PPH is a zinc-dependent enzyme (Chandler et al., 1986). Experimentally induced zinc deficiency in humans caused malabsorption of polyglutamates but not monoglutamates (Tamura et al., 1978). The extent to which variation in zinc nutriture affects the bioavailability of dietary polyglutamates in humans has not been determined. Citric acid may inhibit PPH activity in vitro. The question of whether citric acid in food might potentiate decreased folate bioavailability in humans has not been previously addressed using stable isotope methodology. Bhandari and Gregory (1990) indicated that orange juice, legumes, and tomatoes inhibited the jejunal brush border membrane PPH activity in human and porcine in vitro. Whether these foods inhibit folate bioavailability in vivo was the focus of this research. Because subjects were given 2 mg folate per day during the trials to maintain the folate saturation regimen, serum and erythrocyte folate concentrations are much higher than acceptable (low risk) range (≥6.0 ng/mL for serum, ≥160 ng/mL for red blood cell; Sauberlich, 1977) and stayed consistent during the six trials (Tables 11 and 12).

Effects of selected food extracts, including orange juice, tomato juice, and lima beans, and 52 mM citric acid on folate excretion in urine of human subjects during the trials are shown in Table 13. The urinary folate excretions in human subjects during six trials are around 1 µmol/d. For some reason the subjects in trial 6 had somewhat lower urinary folate excretion than in other trials.

The percentage recovery of d_2-PteGlu$_6$ dose as urine d_2-folate for human subjects during the trials is shown in Table 17 and Figure 33. There are no significant differences between the control trial and tested food trials (power=0.773). Table 18 and Figure 34 list the percentage recovery of d_4-PteGlu$_1$ dose as urine d_3- plus d_4-folate for human subjects during the trials. There are no significant differences between control trial and tested food trials (power=0.64). The powers of the performed tests (0.773 and 0.64) are below the desired power of 0.8 which means that the interpretation of the negative findings should be very cautiously. These low powers are due to small subject numbers and could be increased by increasing subject numbers. The use of the calculated excretion ratio permitted an assessment of the relative bioavailability of the polyglutamyl folate in a manner that corrected for variation in overall folate absorption among subjects and trials. Table 19 and Figure 35 list the urinary excretion ratio of pteroylpolyglutamate vs pteroylmonoglutamate for the human subjects during the trials. A ratio of around one indicates that both poly- and monoglutamates have similar absorption. A ratio lower than one indicates that polyglutamate and monoglutamate have differences in absorption which can only be due to decreased deconjugation of polyglutamates.

In control (trial 1) the $d_2/(d_3+d_4)$ excretion ratio was 1.14±0.17 (Mean±SD). This indicates that d_2-PteGlu$_6$ and d_4-PteGlu$_1$ exhibited similar absorption and excretion patterns. No significant difference between the two controls (1.14±0.17 vs 1.05±0.14) indicates that there was no change in efficiency of polyglutamate absorption during this long period. The $d_2/(d_3+d_4)$ excretion ratio in the orange juice trial was lower than the control (0.66±0.28), which reflected a lower absorption of d_2-PteGlu$_6$ relative to d_4-PteGlu$_1$ ($P < 0.05$) which due to less deconjugation of d_2-PteGlu$_6$. The ratios in the tomato juice trial (0.95±0.13), the lima beans trial (0.97±0.27), and citrate trial (0.86±0.16) were close to the two control trials (1.14±0.17 and 1.05±0.14). These results indicate that tomato juice, lima beans, and citric acid did not significantly influence the relative extent of d_2-PteGlu$_6$ deconjugation. These results suggest that orange juice caused incomplete bioavailability of this deuterated polyglutamyl folate in humans.

The saturable mode of folate absorption is markedly influenced by pH, with maximum absorption at pH 6.3 and a sharp reduction between 6.3 and 7.6 (Russell et al., 1979). Because of the profound influence of intestinal pH on folate absorption, alteration of the pH of the intestinal contents can affect folate absorption. Reduced gastric acid secretion associated with atrophic gastritis in the elderly causes reduced absorption of [^{3}H]folic acid (Russell et al., 1986). Folate absorption in atrophic gastritis could be enhanced by the simultaneous administration of acid, which confirmed a pH effect. An opposite effect of intestinal pH may also occur. Patients with pancreatic insufficiency exhibited greater folate absorption than normal controls because of their slightly acidic intestinal pH (near the optimum for folate absorption) because of insufficient pancreatic bicarbonate secretion (Russell et al., 1979). The oral administration of phosphate or bicarbonate buffering compounds normalized intestinal pH and reduced folate absorption to the normal range. The results in this study show no significant difference between the control trials and the trials of low pH materials [tomato juice (pH 5.0), citric acid (pH 4.1)] which suggests that the low pH did not reduce the absorption of folates.

In the present study nutritionally relevant amounts of neutralized orange juice caused a strong and concentration-dependent inhibition of porcine jejunal brush border membrane PPH activity in vitro (Figure 9). Sixteen percent of neutralized orange juice completely inhibited porcine PPH activity. These findings indicate that orange juice may affect the deconjugation of the pteroylpolyglutamates in the GI tract in vitro. The $d_2/(d_3+d_4)$ excretion ratio in the orange juice trial was 33% lower than the control, this was attributable to less deconjugation of d_2-PteGlu$_6$.

The in vivo study showed results similar to those of Tamura et al. (1976). Their study employed a single large dose of orange juice with added PteGlu$_1$ or PteGlu$_7$ to folate saturated subjects. The bioavailability of the added PteGlu$_7$ was lower than that of the added PteGlu$_1$, which was attributed to an effect of the large volume of orange juice on intestinal pH, thus inhibiting PPH. However, in the present study there was no significant difference between the orange juice trial and the citric acid trial which employed same pH (4.1). Since, there were no significant inhibitory effects of pH and citrate alone on folate bioavailability. While there was a significant inhibitory effect of orange juice, these findings suggest that the effect of orange juice may be due to the combined effects of pH, citrate, and other anions (ascorbic acid, malic acid, phytic acid, etc.). In vitro K_i values may differ substantially from those occurring in vivo. The results suggest that the organic acids including ascorbic acid, citric acid, malic acid, phytic acid, etc., in food extracts are involved in this inhibition.

SUMMARY AND CONCLUSIONS

Dietary folate exists mainly as polyglutamates ($PteGlu_n$) which require deconjugation by zinc-dependent pteroylpolyglutamate hydrolase (PPH) before intestinal absorption. Since deconjugation by PPH is a major factor governing the absorption of dietary folate, factors influencing the deconjugation process will have an effect on folate bioavailability. Therefore, it is important to understand how these forms are digested and absorbed, and what factors affect these processes. The present study was conducted to evaluate in vitro inhibition of porcine PPH activity by selected foods including orange juice, tomato juice, and lima beans and identify the inhibitors in these foods. Furthermore, the influence of selected foods on folate bioavailability in human subjects was examined using stable-isotope methods with a single-dose, dual-label protocol. The experimental results provide information concerning the effects of food components on the deconjugation and absorption of pteroylpolyglutamates.

Orange juice, tomato juice, and lima beans inhibited porcine PPH activity. Sixteen percent (v/v) of neutralized orange juice in the reaction mixture yielded a complete inhibition in vitro, while the same percentage of neutralized tomato juice caused approximately 70% inhibition. Neutralized lima bean homogenates inhibited porcine PPH much more weakly.

Neutralized whole food extracts as well as the 3kD, 10kD, 30kD, and 100kD filtrate fractions exhibited equivalent inhibition of porcine PPH activity. After dialysis, the neutralized retentates (6~10kD, 6~30kD, 6~100kD) did not significantly inhibit porcine PPH activity. These results indicated that small molecules (MW <6kD) in food extracts were responsible for this inhibition.

Orange juice, tomato juice, and lima beans were fractionated by ion exchange chromatography and the anionic fraction was found to be inhibitory. By using ion chromatography with UV absorbance and conductivity detection to identify and quantify the inhibitors citric acid peak was identified as the major inhibitor in food extracts. Malic acid and phytic acid peaks were only minor inhibitors in the food extracts tested in vitro. The inhibition of porcine PPH activity by orange juice, tomato juice, lima beans, and selected organic acids including ascorbic acid, citric acid, malic acid, and phytic acid appeared to be competitive with respect to $PteGlu_3$.

The reversibility of inactivation of porcine PPH activity was studied by increasing extra zinc to reverse the inhibition caused by food extracts. The inhibition of PPH activity was not reversed by providing additional zinc acetate in the reaction as high as 700 μM. The results indicated the inhibition was not mediated by a zinc-chelation process.

Folate bioavailability in human subjects was examined using stable-isotope methods with a single-dose, dual-label protocol. The labeled compounds were [3',5'-$^2H_2$]pteroylpolyglutamate (d_2-$PteGlu_6$) and [glu-$^2H_4$]pteroylmonoglutamte (d_4-$PteGlu_1$). In six trials, adult males (n=7) on a folate saturation regimen (2 mg/d) were given a single

677 nmol oral dose of d_2-PteGlu$_6$ and d_4-PteGlu$_1$ in food extracts including orange juice, tomato juice, lima beans, and 52 mM citric acid, with water as the control. Urine was collected for 48 h and the isotopic labeling of urinary folates determined by gas chromatography-mass spectrometry. The d_2/d_4 isotope ratios of urinary folate were used as criteria for the relative bioavailability of the pteroylmonoglutamates vs. pteroylpolyglutamates. The d_2/d_4 excretion ratio of urinary folate resulting from selected food extracts was less than control value of 1.14±0.17 (Mean±SD). This indicates that orange juice partially inhibited the utilization of the labeled polyglutamyl folate. These results indicate that components of orange juice might impair deconjugation of polyglutamyl folate and thus reduce the bioavailability of its endogenous folate.

Since, there were no significant inhibitory effects of pH and citrate alone on folate bioavailability. While there was a significant inhibitory effect of orange juice, these findings suggest that the effect of orange juice may be due to the combined effects of pH, citrate, and other anions (ascorbic acid, malic acid, phytic acid, etc.). In vitro K_i values may differ substantially from those occurring in vivo. The results suggest that the organic acids including ascorbic acid, citric acid, malic acid, phytic acid, etc., in food extracts are involved in this inhibition.

The major hypotheses of this study are that factors that influence the polyglutamate deconjugation be PPH will have inhibition on folate absorption and bioavailability. The results of this study have indicated that components having molecular weight less than 6kD in the anionic fractions, including ascorbic acid, citric acid, malic acid, and phytic acid, were responsible for the inhibition of porcine PPH activity. This inhibition was not mediated by a Zinc-chelation process. Orange juice partially impairs deconjugation of polyglutamyl folate and, thus partially reduces folate bioavailability in humans. However, orange juice is rich in folates. When considering both folate content and the incomplete folate bioavailability in orange juice, orange juice is an excellent source of available folate.

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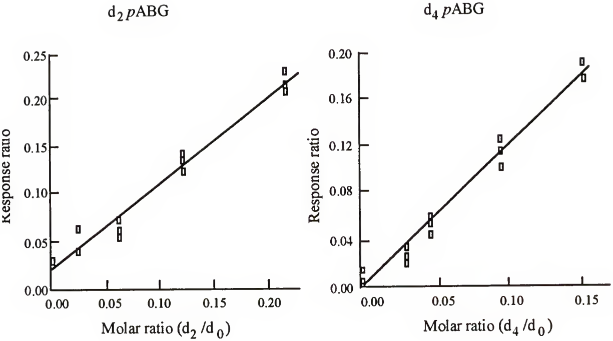
APPENDIX A

Gas Chromatography/Mass spectrometry:

Standard Curve

Source from John P. Toth, Ph.D.



Standard curves of d_2 *p*ABG and d_4 *p*ABG from the GC/MS.

APPENDIX B

Gas chromatography/Mass spectrometry:

Simultaneous Equations

Source from John P. Toth, Ph.D.

Molar ratios of isotopically labeled species were calculated from relative intensities at m/z 427, 428, 429, and 430 using the following 1~4 equations:

1. $I'_{427} = C_{10} + X'_{d1}$

2. $I'_{428} = C_{20} + C_{21}X'_{d1} + X'_{d2}$

3. $I'_{429} = C_{30} + C_{31}X'_{d1} + C_{32}X'_{d2} + X'_{d3}$

4. $I'_{430} = C_{40} + C_{41}X'_{d1} + C_{42}X'_{d2} + C_{43}X'_{d3} + X'_{d4}$

Where X'_{dj} = the molar ratio of the species labeled with j deuterium atoms to the unlabeled species:

$X'_{d1} = n_{d1}/n_{d0}$

$X'_{d2} = n_{d2}/n_{d0}$

$X'_{d3} = n_{d3}/n_{d0}$

$X'_{d4} = n_{d4}/n_{d0}$

Where n is the number of moles of each species.

I'_n represents the intensity of the signal at m/z = n relative to that at m/z = 426. In these experiments intensities were measured as chromatographic peak area A in each single-ion mass chromatograms:

$I'_{427} = A_{427}/A_{426}$

$I'_{428} = A_{428}/A_{426}$

$I'_{429} = A_{429}/A_{426}$

$I'_{430} = A_{430}/A_{426}$

The coefficients C_{ij} represent the contribution of species j at the m/z value corresponding to the species with i deuterium labels:

C_{10} = intensity at m/z 427 due to the presence of ^{13}C, ^{15}N, and ^{17}O in the unlabeled species.

C_{20} = intensity at m/z 428 due to the presence of ^{13}C, ^{15}N, ^{17}O and ^{18}O in the unlabeled species.

etc.

The coefficients C_{ij} can be calculated from the theoretical intensities based on the natural abundances of the ^{13}C, ^{15}N, and ^{17}O.

e.g., $C_{20} = I(^{13}C_2) + I(^{13}C_1{}^{17}O_1) + I(^{13}C_1{}^{15}N_1) + I(^{17}O_2) + I(^{15}N_2) + I(^{18}O_1)$

where $I(^{13}C_2)$ = intensity at m/z 428 due to molecules with two ^{13}C atoms, $I(^{13}C_1{}^{17}O_1)$ = intensity at m/z 428 due to molecules with one ^{13}C atom and one ^{17}O atom, etc. These are calculated from the known natural abundances of the isotopes and the number of each type of atom in the molecule (16 carbons, 2 nitrogens, and 5 oxygens).

The calculated theoretical values of the coefficients are :

$C_{10} = C_{21} = C_{32} = C_{43} = 0.1854$

$C_{20} = C_{31} = C_{42} = 0.02619$

$C_{30} = C_{41} = 0.00347$

C_{40} negligible

In practice, experimental values of C_{ij} were determined by observing the relative intensities at m/z 427, 428, 429, and 430 in an unlabeled standard. These experimental values always matched the theoretical values with an error of 10% or less, with the differences attributable to the instrumental conditions (depending on mass resolution and symmetry, some intensity at m/z 426 may be assigned to m/z 427, etc.). Coefficients used in calculating the molar ratios were determined from unlabeled standards run within one day of the samples.

Equations 1~4 were solved for X'_{dn} values via standard matrix inversion and multiplication performed with the Microsoft Excel spreadsheet program.

BIOGRAPHICAL SKETCH

Ming-Min Wei was born on February 3, 1962, in Taiwan. She received a Bachelor of Science degree in health and nutrition from Taipei Medical College in June, 1985. Then she worked as a teaching assistant in the Department of Anatomy, National Yang-Ming Medical College. Two years later she became a graduate student and research assistant at the Institute of Neuroscience, National Yang-Ming Medical College. After a two-year study she achieved a master's degree with her thesis titled "Studies of Bradykinin Receptor Subtypes and the Involvement of Cyclic Nucleotides in the Receptor-Mediated Response" in June, 1989. She entered the Ph.D. program in the Food Science and Human Nutrition Department at the University of Florida, Gainesville, in the fall of 1991. In December, 1994 she graduated with the degree of Doctor of Philosophy.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jesse F. Gregory III

Jesse F. Gregory, III, Chairman
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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December, 1994

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